



BEST AVAILABLE COPY

Express Mail No.: EV 452 775 121 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | | | |
|------------------|---|----------------------|--------------------|
| Application of: | Soff <i>et al.</i> | Confirmation No.: | 2549 |
| Application No.: | 09/500,397 | Art Unit: | 1642 |
| Filed:: | February 8, 2000 | Examiner: | Davis, Minh Tam B. |
| For: | METHODS AND COMPOSITIONS FOR GENERATING ANGIOSTATIN | Attorney Docket No.: | 10561-005-999 |

DECLARATION OF GERALD A. SOFF, M.D., UNDER 37 C.F.R. § 1.132

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

GERALD A. SOFF, M.D., declares and states that:

1. I am a citizen of the United States, residing at 303 Appletree Lane, Wilmette, IL 60091.

2. I presently hold the position of Associate Professor of Medicine at Northwestern University Medical School, Chicago, IL, which position I have held since 2000. From 1991 to 2000, I held the position of Assistant Professor of Medicine, Northwestern University Medical School, Chicago, IL. I have held the positions of: Instructor in Medicine, Beth Israel Hospital, Harvard Medical School, Boston, MA; Visiting Scientist, Massachusetts Institute of Technology, Cambridge, MA; and Fullbright Scholar, Council of International Exchange of Scholars.

3. I received a B.A. from Johns Hopkins University in 1977 and an M.D. from Johns Hopkins School of Medicine in 1981. I completed a three-year internship/residency in medicine at the Medical College of Virginia and a three-year fellowship in hematology/oncology at Beth Israel Hospital, Harvard Medical School, Boston, MA. I am a Diplomat of The American Board of Internal Medicine, Subspecialty in Hematology.

4. My academic and technical experience, honors, and a list of my publications are set forth in my *curriculum vitae*, attached hereto as Exhibit A.

5. I am a co-inventor of present U.S. Patent Application No. 09/500,037, filed February 8, 2000, in the name of Soff *et al.* and entitled "Methods and Compositions for Generating Angiostatin", and a co-author of Gately *et al.*, The mechanism of cancer-mediated conversion of plasminogen to the angiogenesis inhibitor angiostatin. Proc Natl Acad Sci U S A. 1997;94(20):10868-72 ("Gately"), attached hereto as Exhibit B, and Lannutti *et al.*, Human angiostatin inhibits murine hemangioendothelioma tumor growth in vivo. Cancer Res. 1997 Dec 1;57(23):5277-80 ("Lannutti"), attached hereto as Exhibit C. I am familiar with the non-final Office Action dated October 20, 2004 ("Office Action") issued therein.

6. In the Office Action, the Examiner rejected claims 19-21, 23-24, 76-77, 79-86 and 88-90 based on a lack of enablement, stating that applicant has not taught that plasminogen activator is effective in treating any angiogenic disease, and in particular hemangiomas (see Office Action at page 4, ¶5).

7. The following experiments have been performed. The results demonstrate that (1) administration of a plasminogen activator alone to an animal increases the amount of angiostatin present in said animal; and (2) the increased amount of angiostatin is effective to treat hemangiomas in the animal.

A. Plasminogen Activator Alone Converts Plasminogen to Angiostatin in Ex Vivo Plasma

8. An experiment was conducted to demonstrate that a plasminogen activator alone is effective to generate angiostatin in human plasma *ex vivo*.

9. A plasminogen activator (uPA) and/or a free sulfhydryl donor (captopril) was added to 1 ml of normal human plasma anticoagulated with 10 mM EDTA. As control, plasma was incubated with no reagents. Plasma was incubated at 37°C, from 0 to 18 hours for analysis on immunoblot.

10. Plasma treated with both uPA (200 IU/ml) and captopril (50 µM) resulted in the generation of Angiostatin4.5 ("AS4.5") (see Figure 1 in Exhibit 1). Based on immunoblot and densitometer, it was estimated that the combined treatment resulted in the generation of approximately 100 nM of Glu-AS4.5 and 200 nM of Lys-AS4.5.

11. Plasma treated with captopril alone did not result in the generation of AS4.5 (see Figure 1 in Exhibit 1). However, plasma treated with uPA alone did result in the generation of AS4.5 (see Figure 2 in Exhibit 2). Based on immunoblot and densitometer, it

was estimated that the treatment with the plasminogen activator alone resulted in the generation of approximately 20-40 nM of Lys-AS4.5 (see Figure 1 in Exhibit 1).

12. Thus, the results demonstrate that a plasminogen activator alone is effective to generate angiostatin in human plasma by first converting plasminogen to plasmin, which is then converted to angiostatin in the presence of a sulfhydryl donor that is either endogenously present (see Figure 2 in Exhibit 2) or added to the plasma *ex vivo* (see Figure 1 in Exhibit 1).

B. Angiostatin Generated by the Administration of a Plasminogen Activator Alone is Effective to Treat Hemangiomas in Mice

13. An experiment was previously conducted to demonstrate that plasminogen activator-generated angiostatin is effective to treat hemangiomas (see Lannutti).

14. The plasminogen activator uPa was capable of generating angiostatin in the presence of endogenous sulfhydryl donor (see Gately).

15. Female beige nude mice at 6-8 weeks of age were first injected s.c. with cells from the murine hemangioendothelioma cell line EOMA and then, after 24 hours, injected s.c. with the affinity-purified plasminogen activator-generated human angiostatin twice daily ("angiostatin-treated mice") or PBS ("control mice") until sacrifice. The mice were weighted every other day, and the tumors were measured three times weekly using tissue calipers.

16. By 10 days after tumor appearance, the mean volume of tumors in the angiostatin-treated mice was significantly less than the volume of tumors in the control mice (see Figure 1 in Lannutti).

17. The control mice were euthanized between 15 and 20 days after inoculation when preterminal, and the angiostatin-treated mice were euthanized at 20 days after inoculation. Blood, spleens and tumors were collected at autopsy and weighed. The wet weight of tumors in the control mice was significantly higher than that in angiostatin-treated mice (see Figure 2A in Lannutti).

18. The spleen weight of angiostatin-treated mice was not significantly higher than age-matched control mice without hemangioendothelioma. By contrast, the spleen weight of control mice with hemangioendothelioma was significantly higher than non-tumor-bearing control mice (see Figure 2B in Lannutti).

19. The platelet count in angiostatin-treated mice was not significantly lower than non-tumor-bearing control mice but was significantly lower in the control mice with hemangioendothelioma (see Figure 2C in Lannutti).

20. The hematocrit level in angiostatin-treated mice was almost the same as those in non-tumor-bearing control mice. In comparison, the hematocrit level in the control mice with hemangioendothelioma were significantly reduced (see Figure 2D in Lannutti).

21. Thus, the results demonstrate that angiostatin generated by the administration of a plasminogen activator alone is effective to treat hemangiomas in mice.

22. As discussed above, a plasminogen activator converts plasminogen to plasmin, which is then converted to angiostatin in the presence of a sulfhydryl donor that can be from an endogenous source or added from an external source (see specification, page 10, lines 11-14). The sulfhydryl donor from an endogenous source is present in an amount that is sufficient to convert the intermediate plasmin to angiostatin (see ¶11 and ¶14). The resulting increase in the amount of angiostatin in the body is effective to treat an angiogenic disease, such as hemangiomas (see ¶¶15-21).

23. In view of the foregoing, I conclude and it is my opinion that one skilled in the art would conclude that plasminogen activator-generated angiostatin is effective for treating an angiogenic disease, such as hemangiomas. The results demonstrate that when administered alone, the dose and dosage regimen of plasminogen activator can be adjusted to generate levels of angiostatin that have a clinical benefit in patients with hemangiomas.

24. In view of the foregoing, I also conclude and it is my opinion that others skilled in the art would conclude that the present specification provides enablement for methods of treating an angiogenic disease, such as hemangiomas, comprising administering to an animal suffering from said disease a plasminogen activator, wherein the plasminogen activator converts plasminogen to plasmin, which is then converted to angiostatin by endogenous sulfhydryl donor.

25. I declare further that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,

A handwritten signature in black ink, appearing to read "Gerald Soff", written in a cursive style.

Date: 4-20-2005

Gerald A. Soff, M.D.

CURRICULUM VITAE

Gerald A. Soff MD

Address: 303 Apple Tree Lane
Wilmette, IL 60091

Date of Birth: October 12, 1955 Soc. Security; 058 44 4104

Place of Birth: New York, New York

Education:

1977 B.A. Cornell University (1973-1975) and Johns Hopkins University (1975-1977)
1981 M.D. Johns Hopkins School of Medicine

Postdoctoral Training:

Internship/Residency:

1981-1984 Medicine, Medical College of Virginia

Fellowship.

1985-1988 Hematology/Oncology, Beth Israel Hospital,
Harvard Medical School, Boston, MA.

Licensure and Certification:

1982 Virginia Medical License #0101034414

1986 Massachusetts Medical License #56474

1991 Illinois State Medical License #036-083173

DEA # BS 0748333

Board Certification:

1984 Diplomat of The American Board of Internal Medicine.

1988 Diplomat of The American Board of Internal Medicine,
Subspecialty in Hematology.

Academic Appointments/Previous Positions

1984-1985 Fulbright Scholar,
Council for International Exchange of Scholars.

1988-1991 Instructor in Medicine, Beth Israel Hospital,
Harvard Medical School, Boston, MA.

1986-1991 Visiting Scientist, Massachusetts Institute of Technology,
Cambridge, MA.

1991- 2000 Assistant Professor of Medicine, Northwestern University Medical School,
Chicago, IL.

Academic Appointments/Current Position

2000- Associate Professor of Medicine, Northwestern University
Medical School, Chicago, IL.

Patents

1. "A method of generating angiostatin *in vitro* comprising contacting plasminogen or plasmin with a plasminogen activator and a sulfhydryl donor." US Patent # 5,801,012. Issued September 1, 1998.

2. "Methods and compositions for generating angiostatin" US Patent #6,576,609 B1. Issued June 10, 2003.

Awards and Honors:

- 1978-1979 Henry Strong Denison Scholarship recipient
of the Johns Hopkins University School of Medicine.
1984-1985 Fulbright Scholar,
Council for International Exchange of Scholars.

Major Committee Assignments/Hospital:

- 1998- Medical Director, Anticoagulation Dosing Service,
Northwestern Memorial Hospital
1998- Member, Pharmacy And Therapeutics Committee,
Northwestern Memorial Hospital
1996-2003 Medical Director of The "Rube Walker" Blood Center, at
Northwestern Memorial Hospital
1995-1996 Assistant Director of Rube Walker Blood Center, at
Northwestern Memorial Hospital

Major Committee Assignments/ Medical School:

- 1991-1997 Member of Northwestern University Intramural Grant Committee
1996-1997 Chairman, Northwestern University Intramural Grant Committee
2000- Present; Member, Northwestern Memorial Foundation Intramural Grant Committee
2002- present; Chair, Northwestern Memorial Foundation Intramural Grant Committee
2005- present; Program Director; Medical School Honors In Research Program

Membership In Professional Organizations

- 1993 Member of American Society of Hematology.
1997 American Association For Cancer Research
1997 Central Society For Clinical Research

Major Research Interests:

- 1) Angiogenesis
- 2) Thrombosis and Hemostasis
- 3) Vascular biology

Mentoring Research Trainees:

Mentored Medicine Residents In Research Electives

- 1993; Serena Yoon M.D.; First Prize in Residents Research Symposium
1995; Lisa Boggio M.D.; First Prize in Residents Research Symposium.
1997; Suzie Chi M.D.; First Prize in Residents Research Symposium.

1997- 1998; Douglas Tomasian M.D.; First Prize in Residents Research Symposium.
1998; Anne Mellott M.D.;
1998; Philip Simonian M.D.
2000; John Eklund MD
2000; Dan Milton MD
2002; Alexandra Leary MD
2003; Denise Lo (Medical Student); Medical Student Research Award
2003; Monica Tripathi MD

Mentored Hematology Oncology Fellows In Research Programs

1993-1995; Judith Senderowicz M.D. (Deceased)
1993-1995; Ann Traynor M.D. (Currently faculty at Un. Massachusetts)
1994-1995; Elaine Lee Wade M.D.
1995-1997; Przemyslaw Twardowski M.D. (Currently, Staff Of City Of Hope Medical Center)
1997- 1999; Lisa Boggio M.D. (Currently Instructor at Northwestern University)
1997- 1999; Jerome Hong M.D.
2002 - 2004; Anaa Zakarija M.D. (Currently Instructor at Northwestern University)

Mentored Post-Doctoral Research Fellows

1994-1998; Stephen Gately Ph.D.
2003-present; Jennifer Doll PhD

Mentored Graduate Students/PhD Advisor

1999 - 2004: Hao Wang PhD
2002 - present: Jennifer Hobbs

Principal Clinical and Hospital Service Responsibilities:

Attend on benign Hematology consult service, 4 months per year.
Medical Director of;

- 1) Anticoagulation Dosing Service, Northwestern Memorial Hospital
- 2) The Blood Center, Northwestern Memorial Hospital

GRANT SUPPORT

Current Grant Support

1) NIH, (P50 CA90386) The Specialized Program Of Research Excellence- Prostate Cancer
Project Title; Generation of, And Angiostatin Levels In Prostate Cancer

Dates; 06/01/01 – 04/30/06

Direct Costs for current year; \$123,678

2) NIH (P50 CA89018-02), The Specialized Program Of Research Excellence-Breast Cancer
Project Title; Angiostatic Therapy For Breast Cancer: A Translational Study

Dates; 09/30/00 – 8/31/05

Direct Costs for current year; \$138,846

3) NIH, R21 CA 89886-01

Title; *In Vivo* Generation of Angiostatin^{4,5} Clinical Trial

P.I.; Timothy Kuzel MD. CO-P.I.; Gerald A. Soff MD

Dates; 7/1/01 – 6/30/03

Direct Costs for current year; \$225,000

4) Hairy Cell Leukemia Research Foundation

Title; Mechanism of Antiangiogenic Activity of Angiostatin^{4,5}.

Dates; June, 2002- June, 2003

Direct Costs for current year; \$20,000

Previous Grant Support

1) Clinician Scientist Award From American Heart Association

Title; Regulation of the Gene For thrombomodulin

P.I.; Gerald Soff M.D.

Dates 7/1/1989- 6/30/1992

2) Grant-In-Aid From American Heart Association of Metropolitan Chicago Title; Cyclic Nucleotide-Dependent gene Regulation In Smooth Muscle Cells P.I.; Gerald Soff M.D.

Dates; 7/1/1992 – 6/30/1994

3) Grant-In-Aid From American Cancer Society, Illinois Division Title; u-PA In The Microenvironment And Metastasis Of Prostate Cancer P.I.; Gerald Soff M.D.

Dates; 8/1/1994 – 7/31/1995

4) Grant-In-Aid From The Feinberg Institute For Cardiovascular Research

Title; Regulation of Cyclic GMP-Dependent Protein Kinase In Vascular Smooth Muscle Cells

P.I.; Gerald Soff M.D.

Dates; 3/1/1994 – 2/28/1995

Renewal; 7/1/1995 – 6/30/ 1996

5) RO1 from NIH

Title; The Vasculopathy of Juvenile Dermatomyositis

P.I.; Lauren Pachman M.D.

Co-P.I.; Gerald Soff M.D.

Dates 9/30/95 – 9/29/97

Direct Costs per year (For Soff); \$40,188 Indirect Costs for per year (For Soff); \$ 19,290

6) RO1 (RO1 CA71875) NIH/NCI

Title; Plasminogen/Angiostatin Converting Enzyme

P.I.; GA Soff

Dates; 4/1/1997- 1/32/2001

Direct Costs for current year; \$153,355

Indirect Costs for current year; \$73,610

7) RO1 from NIH/NHLBI

Title; Oral Contraceptives and Thromboembolic Disease

P.I.; Stephen Sidney M.D.

Co-P.I.; Gerald Soff M.D.

Dates 7/1/1997 – 6/30/2001

Direct Costs per year (For Soff); \$19,560

Indirect Costs per year (For Soff); \$ 7997

BIBLIOGRAPHY

Original Reports:

1. **Soff GA**, Levin J. Familial multiple coagulation factor deficiencies. I. Review of the literature: Differentiation of single hereditary disorders associated with multiple factor deficiencies from coincidental concurrence of single factor deficiency states. *Semin Thromb Hemostas*. 7:112-148, 1981.
2. **Soff GA**, Levin J, Bell WR. Familial multiple coagulation factor deficiencies. II. Combined factor VIII, IX, and XI deficiency and combined factor IX and XI deficiency: Two previously uncharacterized familial multiple factor deficiency syndromes. *Semin Thromb Hemostas*. 7:149-169, 1981.
3. **Soff GA**, Sica DA, Marlar RA, Evans HJ, Qureshi GD. Protein C levels in nephrotic syndrome. Use of a new enzyme-linked immunoadsorbent assay for protein C antigen. *Am J Hemat*. 22:43-49, 1986.
4. Margulis T, David M, Maor N, **Soff GA**, Grenadier E, Palant A, Aghai E. The von Willebrand factor in myocardial infarction and unstable angina: a kinetic study. *Thromb Haemost*. 55:366-368, 1986.
5. **Soff GA**, Kadin ME. Tocainide-induced reversible agranulocytosis and anemia. *Arch Intern Med*. 147:598-599, 1987.
6. Weiss P, **Soff GA**, Halkin H, Seligsohn U. Decline of proteins C and S and factors II, VII, IX, and X during the initiation of warfarin therapy. *Thrombosis Res*. 45:783-790, 1987.
7. Jackman RW, Beeler DL, Fritze L, **Soff GA**, Rosenberg RD. The human thrombomodulin gene is intron depleted: Nucleic acid sequences of the cDNA and gene predict protein structure and sites of regulatory control. *Proc Nat Acad Sci. (USA)* 84:6425-6429, 1987.
8. **Soff GA**, Levin J. Thrombocytopenia associated with repletion of iron in iron-deficiency anemia. *Am J Med Sci* 295:35-39, 1988.
9. **Soff GA**, Jackman RW, Rosenberg RD. Expression of thrombomodulin by smooth muscle cells in culture. (Different effects of tumor necrosis factor and cyclic AMP on thrombomodulin expression by endothelial cells and smooth muscle cells in culture). *Blood* 77(3):515-518, 1991.
10. Weiler-Guettler H, Yu K, **Soff G**, Gudas LJ, Rosenberg RD. Thrombomodulin gene regulation by cAMP and retinoic acid in F9 embryonal carcinoma cells. *Proc Nat Acad Sci. (USA)* 89:2155-2159, 1992.
11. **Soff GA**, Green D. Autoantibody to von Willebrand factor in systemic lupus erythematosus. *J. Lab. Clin. Med.*, 121:424-430, 1993.
12. Cornwell TL, **Soff GA**, Traynor AE, Lincoln TM. Regulation of the expression of cyclic GMP-dependent protein kinase by cell density in vascular smooth muscle cells. *J. Vasc. Res*. 31:330-337, 1994.

13. Traynor AE, Cundiff D, **Soff GA**. cAMP influence on transcription of thrombomodulin and myogenic proteins in smooth muscle is dependent on de novo synthesis of a protein intermediate. *J. Lab. Clin. Med.* 126:317-323, 1995.
14. **Soff GA**, Senderowicz J, Gately, S., Verrusio E, Weiss I, Brem S, Kwaan HC. Expression of Plasminogen Activator Inhibitor Type 1 By Human Prostate Carcinoma Cells Inhibits Primary Tumor Growth, Tumor-Associated Angiogenesis, And Metastasis To Lung And Liver In An Athymic Mouse Model. *J. Clin. Invest.* 96:2593-2600, 1995.
15. Gately S, **Soff GA**, Brem S. The potential role of basic fibroblast growth factor (bFGF) in the transformation of cultured primary human fetal astrocytes and the proliferation of human glioma (U-87) cells. *Neurosurgery* 37:723-732, 1995.
16. Gately S, Twardowski P, Stack MS, Patrick M, Boggio L, Cundiff DL, Schnaper HW, Madison L, Volpert O, Bouck N, Enghild J, Kwaan HC, **Soff GA**. Human prostate carcinoma cells express enzymatic activity that converts human plasminogen to the angiogenesis inhibitor, angiostatin. *Cancer Research* 56:4887-4890, 1996.
17. Green D, Maliekel K, Sushko E, Akhtar R, **Soff GA**. Activated-protein-C resistance in cancer patients. *Haemostasis* 27:112-118, 1997.
18. Gately S, Twardowski P, Stack MS, Cundiff DL, Grella D, Castellino FJ, Enghild J, Kwaan HC, Lee F, Kramer RA, **Soff GA**. The Mechanism of Cancer-Mediated Conversion of Plasminogen to the Angiogenesis Inhibitor, Angiostatin. *Proc Nat Acad Sci. (USA)*, 94:10868-10872, 1997.
19. **Soff GA**, Cornwell TL, Cundiff DL, Wade EL, Lincoln TM. Smooth Muscle Cell Expression Of Type I Cyclic GMP-Dependent Protein Kinase Is Suppressed By Continuous Exposure To Nitrovasodilators, Theophylline, cyclic GMP, and cyclic AMP. *J Clin Invest.* 100:2580-2587, 1997.
20. Lannutti BJ, Gately ST, Quevedo ME, **Soff GA**, Paller AS. Human angiostatin inhibits murine hemangioendothelioma tumor growth *in vivo*. *Cancer Research* 57:5277-5280, 1997.
21. Mauceri HJ, Hanna NN, Beckett MA, Staba M-J, Gorski DH, Bigelow K, Heinmann R, Gately S, **Soff GA**, Kufe DW, Weichselbaum RR. Angiostatin and Radiation Target the Tumor Vasculature. *Nature* 394:287-291, 1998.
22. Lucas R, Holmgren L, Garcia I, Jimenez B, Mandriota SJ, Borlat F, Sim BK, Wu Z, Grau GE, Shing Y, **Soff GA**, Bouck N, Pepper MS. Multiple forms of angiostatin induce apoptosis in endothelial cells. *Blood* 92: 4730-41, 1998.
23. Gorski DH, Mauceri HJ, Salloum RM, Gately S, Hellman S, Beckett MA, Sukhatme VP, **Soff GA**, Kufe DW, Weichselbaum RR. Potentiation of the antitumor effect of ionizing radiation by brief concomitant exposures to angiostatin. *Cancer Research* 58: 5686-5689, 1998.
24. Lincoln TM, Dey NB, Boerth NJ, Cornwell TL, **Soff GA**. Nitric oxide--cyclic GMP pathway regulates vascular smooth muscle cell phenotypic modulation: implications in vascular diseases. *Acta*

Physiol Scand 164: 507-15, 1998.

25. Stack MS, Gately S, Bafetti LM, Enghild JJ, **Soff GA**. Angiostatin inhibits endothelial and melanoma cellular invasion by blocking matrix-enhanced plasminogen activation. *Biochem J*. 340: 77-84, 1999.
26. Gohongi T, Fukumura D, Boucher Y, **Soff GA**, Todoroki T, Jain RK. Suppression of angiogenesis at a second site by an orthotopic gallbladder tumor: Role of TGF-1. *Nature Medicine* 5:1203-1208, 1999.
27. Gradishar WJ, **Soff G**, Liv J, Cisneros A, French S, et al. A pilot trial of suramin in metastatic breast cancer to assess antiangiogenic activity in individual patients. *Oncology* 58: 324-333, 2000.
28. Mehta J, Oyama Y, Winter J, Williams S, Tallman M, Singhal S, Villa M, Shook T, Burt R, Traynor A, **Soff G**, Masarik S, Ramsey G, Gordon L. CD34(+) cell collection efficiency does not correlate with the pre-leukapheresis hematocrit. *Bone Marrow Transplant* 28:597-601, 2001.
29. Sellak H, Yang X, Cao X, Cornwell T, **Soff GA**, and Lincoln T. Zinc finger transcription factor Sp1 as a molecular target for nitric oxide-and cyclic nucleotide-mediated suppression of cGMP-dependent protein kinase-I α expression in vascular smooth muscle cells. *Circulation Research* 90:405-412, 2002.
30. Bai, X, Cerimele F, Ushio-Fukai M, Waqas M, Campbell PM, Govindarajan B, Der, CJ, Battle T, Frank DA, Ye K, Murad E, Dubiel W, **Soff G**, and Arbiser JL. Honokiol, a small molecular weight natural product, inhibits angiogenesis in vitro and tumor growth in vivo. *J Biol Chem*, 278: 35501-35507, 2003.
31. Hanford HA, Wong CA, Sandusky H, Cundiff D, Chandel N, **Soff GA**. Angiostatin4.5-mediated apoptosis of vascular endothelial cells. *Cancer Research*.63:4275-4289, 2003.
32. Wang H, Schultz R, Hong J, Cundiff DL, Jiang K, and Soff GA. Cell surface-dependent generation of angiostatin4.5. *Cancer Research*, 64: 162-168, 2004.
33. Sidney S, Petitti DB, Soff GA, Cundiff DL, Tolan KK, Quesenberry CP Jr. Venous thromboembolic disease in users of low-estrogen combined estrogen-progestin oral contraceptives. *Contraception* 70:3-10, 2004.
34. Soff, GA, Wang, H, Cundiff, DL, Jiang, K, Rossbach, HC, Martone, B, and Kuzel, TM. *In Vivo* Generation of Angiostatin4.5 By An Angiostatic Cocktail In Patients With Refractory Cancer (Provisional Acceptance In *Clinical Cancer Research*).
35. Liu, S, Gu, W, Lo, D, Ding, X-Z, Ujiki, M, Adrian, TE, Soff, GA, Silverman, RB. *N*-Methylsalsalvamide A Peptide Analogues. Potent New Antitumor Agents. *Journal of Medicinal Chemistry* (in press), 2005.
36. Wang H, Doll JA, Jiang K, Cundiff DL, Soff GA. Differential binding of plasminogen, plasmin, and angiostatin4.5 to cell surface β -actin: Implications for cancer-mediated angiogenesis.

(Submitted to *Journal of Biological Chemistry*).

Chapters In Textbooks

1. **Soff GA** and Rosenberg RD. Physiology of Coagulation: The Fluid Phase. In Hematology of Infancy and Childhood, DG Nathan and FA Oski (eds.), W.B. Saunders Co., 1993, pp.1534-1560.
2. **Soff GA**. Disseminated Intravascular Coagulation. IN Conn's Current Therapy, RE Rakel (ed.), W.B. Saunders Co., 1993, pp.378-379.
3. **Soff GA**. Thrombotic Thrombocytopenic Purpura. IN Conn's Current Therapy, RE Rakel (ed.), W.B. Saunders Co., 1993, pp.379-381.
4. **Soff GA**. Acquired von Willebrand's Disease. IN Anticoagulants: Physiologic, Pathologic, and Pharmacologic, D Green (ed.), CRC Press, 1994, pp. 157-168.
5. **Soff GA**. Coagulation Mechanisms: Hemostasis and Thrombosis. IN Physiologic and Pharmacologic Bases of Anesthesia, VJ. Collins (ed.), Williams & Wilkins, 1996, pp.214-232.

Invited Reviews:

1. Kwaan HC, **Soff G**. The plasminogen-plasmin system in malignant tumors. *Journal of the Robert H. Lurie Cancer Center of Northwestern University*. 5:15-24, 1996.
2. Kwaan HC, **Soff G**. Management of Thrombotic Thrombocytopenic purpura and Hemolytic Uremic Syndrome. *Seminars in Hematology* 34:159, 1997.
3. **Soff GA**. Angiostatin and angiostatin-related proteins. *Cancer and Metastases Reviews*. 19:97-107, 2000.
4. **Soff GA**. Angiostatin and Hematocellular Carcinoma. *Hepatology* 37(3):696-704, 2003.

Abstracts:

- A1. **Soff GA**, Levin J, Bell WR. Familial multiple coagulation factor deficiencies. *Clin Res*. 29:864A, 1981.
- A2. **Soff GA**. Thrombocytopenia resulting from repletion of iron in iron deficiency anemia. *Virginia Medical Monthly* 110:116, 1983.
- A3. **Soff GA**, Marlar RA, Sica D, Qureshi GD, Evans HJ. Protein C and Protein C inhibitor levels in the nephrotic syndrome. *Clin Res*. 31:875A, 1983.
- A4. **Soff GA**, Weiss P, Halkin H, Griffin JH, Seligsohn U. Half-life of human protein S antigen as determined by a new enzyme-linked immunoadsorbent assay. *Blood* 66 suppl. 1:353a, 1985.
- A5. Beeler D, Fritze L, **Soff G**, Jackman R, Rosenberg R. Human thrombomodulin cDNA: Sequence and translated structure. *Thromb Haemostas*. 320:1168, 1987.

- A6. **Soff GA**, Jackman RW, Rosenberg RD. Expression of thrombomodulin by smooth muscle cells in culture. *Blood* 74 suppl. 1:136a, 1989.
- A7. **Soff GA**, Green D. Autoantibody to von Willebrand factor in systemic lupus erythematosus. *Clinical Research*. 40:425A, 1992.
- A8. Oh D, Kwaan HC, Goolsby C, Verrusio E, Yoshida E, **Soff G**. Urokinase is mitogenic on prostate tumor cell lines. *Blood* 80 suppl. 1:1754a, 1992.
- A9. **Soff GA**, Kwaan HC, Verrusio E, Yoshida, E, Oh DY. Modulation of tumor growth and angiogenesis by PAI-1. *Clinical Research* 43:697a, 1993.
- A9 **Soff GA**, Cundiff DL, Senderowicz J, Cornwell, TL, Traynor, AE. Regulation of The Expression of Cyclic GMP-Dependent Kinase In Vascular Smooth Muscle Cells. *Blood* 82 suppl 1:279a, 1993.
- A10. Traynor AE, Senderowicz J, Cundiff DL, **Soff GA**. Mechanism of cyclic amp stimulation of thrombomodulin expression In Vascular Smooth Muscle Cells. *Blood* 82 suppl 1:279a, 1993.
- A11. Gately S, **Soff GA**, Takano S, Klagsburn M, Brem S. The transforming property of signal peptide-basic fibroblast growth factor (FGF-2) in human astrocytes. *Proc. Am. Assoc. Canc. Res.* 35: 42, 1994.
- A12. Gately S, **Soff GA**, Brem S. Quantitation of basic fibroblast growth factor (bFGF) in human brain tumor tissues by ELISA. *Proc. Am. Assoc. Canc. Res.* 36:24, 1995.
- A13. Gately S, Takano S, **Soff GA**, Brem S. Human glioma cell invasion *in vitro* is correlated with the release of urokinase-type plasminogen activator (u-PA), and decreased expression of cell-associated basic fibroblast growth factor (bFGF). *Proc. Am. Assoc. Canc. Res.* 36:98, 1995.
- A14. Zipfel G, Gately S, **Soff GA**, Engelhard H, Kwaan H, Brem, S. D-penicillamine induces the expression of plasminogen activator inhibitor-1 (PAI-1) mRNA and protein in human glioma cells at clinically relevant concentrations. *Proc. Am. Assoc. Canc. Res.* 36:589, 1995.
- A15. **Soff GA**, Senderowicz J, Takano S., Verrusio E., Weiss I., Brem S., Kwaan HC. PAI-1 expression by human prostate carcinoma cells inhibits primary tumor growth, tumor-associated angiogenesis and metastases in an athymic mouse carcinoma model. *Proc. Am. Assoc. Canc. Res.* 35: 101, 1995.
- A16. **Soff GA**, Gately ST, Twardowski PW, Stack S, Kwaan HC. Identification and characterization of Plasminogen-Angiostatin Converting Enzyme from human prostate carcinoma cells. *Blood* 86 suppl. 1: 742a, 1995.
- A17. **Soff GA**, Gately S, Twardowski P, Stack MS, Boggio LN, Bacallao, R, Kwaan HC. Characterization of Plasminogen-Angiostatin Converting Enzyme from human prostate carcinoma cells. *Proc. Am. Assoc. Canc. Res.* 37:59, 1996.
- A18. **Soff GA**, Twardowski P, Stack MS, Kwaan HC, Gately S. Cancer cell-derived mechanism for

the generation of human angiostatin. *Proc. Am. Assoc. Canc. Res.* 38:#1775, 1997.

A19. Gately S, Twardowski P, Stack MS, Cundiff DL, Grella D, Castellino FJ, Enghild J, Kwaan HC, Lee F, Kramer RA, **Soff GA**. The mechanism of cancer-mediated conversion of plasminogen to the angiogenesis inhibitor angiostatin. *Fibrinolysis & Proteolysis* 11 (Supple. 3) 39, 1997.

A20. Hanna NN, Mauceri HJ, **Soff GA**, Gately S, Beckett MA, Weichselbaum RR. Effect of angiostatin on the growth and radiation response of human cancer xenografts. *Proceedings of the American Society of Surgical Oncology*. 1997.

A21. Gorski D, Mauceri H, Hanna N, Gately S, **Soff G**, Weichselbaum R. Radiation and angiostatin statistically inhibit the growth of Lewis lung carcinoma. *Proceedings of the American Society of Surgical Oncology*. 1997.

A22. **Soff GA**, Hong J, Fishman D, Kleinman M, Kaplan E, Zagzag D, Schultz R, Cundiff D*, Park S, Enghild J, Stack MS, Gately S, Angiostatin4.5: A Naturally occurring human angiogenesis inhibitor. *Blood* 92 (Supple. 1) 174a, 1998.

A23. **Soff GA**, Hong J, Fishman D, Schultz R, Cundiff D, Park S, Enghild J, Stack MS, Gately S. Angiostatin4.5: A Naturally occurring human angiogenesis inhibitor. *Proc. Am. Assoc. Canc. Res.* 40: #4088, 1999.

A24. **Soff GA**, Hoppin EC, Cundiff D, Schultz R, Kunz P. Therapeutic Application of an Angiostatic Cocktail For Patients With Refractory Cancer. *Proc. Am. Assoc. Canc. Res.* 41: #1960, 2000.

A25. Wang H, Hong J, Boggio L, Cundiff D, **Soff GA**. Surface dependence of Plasminogen Conversion To Angiostatin4.5. *Proc. Am. Assoc. Canc. Res.* 41: #3101, 2000.

A26. **Soff GA**, Wong C, Hanford H, Wang H, Cundiff D, French E, Cryns V, Sandusky H, Chandel N. Angiostatin_{4.5} induction of endothelial cell apoptosis. *Proc. Am. Assoc. Canc. Res.* 43:#867, 2002.

A27. Venkatasubramanian PN, **Soff G**, French E, Tom B, Wyrwicz. MRI-Detected heterogeneity of water diffusion in orthotopically implanted murine renal carcinoma. *Proc. Am. Assoc. Canc. Res.* 43: #5224, 2002.

A28. Peaceman A, Kalt S, Casele H, Grobman W, **Soff G**. Fetal death is not associated with an increased incidence of common thrombophilias. *Am J Obstet Gynecol.* 187:S201, 2002.

A29. Lucas, LAG, Mitchell, CA, Prise, V, Wilson, J, Barber, PR, **Soff, GA**, and Tozer, G. An intravitalmicroscopic investigation of the effects of angiostatin4.5 on HT29 tumor vasculature. *Cancer Research*, 44: #1165, 2003.

A30. **Soff GA**, Wang H, Schultz R, Hong J, Cundiff DL, French E, and Jiang K. A role for beta-actin on cancer cell surfaces in plasminogen conversion to angiostatin4.5. *Cancer Research*, 44: #1160, 2003.

A31. Kuzel T, Martone B, Avram A, Rademaker A, **Soff G**. In vivo generation of angiostatin4.5

(AS4.5)-phase I trial of tissue plasminogen activator (tPA) plus mesna. *Proceedings of the American Society of Clinical Oncology*, #879, 2003.

A31. **Soff G**, Wang H, Doll J, Jiang K, Cundiff DL, Differential Binding of Plasminogen And Angiostatin4.5 To Cell Surface Beta-Actin: Implications For Cancer-Mediated Angiogenesis. . *Proceedings of the American Society of Clinical Oncology*, 2004.

A32. **Soff GA**, Wang H, Doll JA, Jiang K, Cundiff DL. Differential Binding of Plasminogen, Plasmin, and Angiostatin4.5 to Cell Surface β -Actin: Implications for Cancer-Mediated Angiogenesis. *Blood* 104 (Supplement, Abstract #1938), 2004.

A33. **McDaniel M**, Soff GA. Decreasing Adverse Drug Events Associated with Lepirudin and Argatroban for the Treatment of Heparin-Induced Thrombocytopenia. *Blood* 104 (Supplement, Abstract #1787), 2004.

The mechanism of cancer-mediated conversion of plasminogen to the angiogenesis inhibitor angiostatin

STEPHEN GATELY*, PRZEMYSŁAW TWARDOWSKI*, M. SHARON STACK†, DEBORAH L. CUNDIFF*, DAVIDA GRELLA‡, FRANCIS J. CASTELLINO‡, JAN ENGHILD§, HAU C. KWAAN¶, FRANCIS LEE||, ROBERT A. KRAMER||, OLGA VOLPERT**, NOEL BOUCK**, AND GERALD A. SOFF*††

*Department of Medicine, Division of Hematology/Oncology, †Department of Obstetrics and Gynecology, **Department of Microbiology-Immunology and R. H. Lurie Cancer Center, Northwestern University School of Medicine, Chicago, IL 60611; ‡Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556; §Department of Pathology, Duke University, Durham, NC 27710; and ¶Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 08543

Communicated by Laszlo Lorand, Northwestern University Medical School, Chicago, IL, August 7, 1997 (received for review June 23, 1997)

ABSTRACT Angiostatin, a potent naturally occurring inhibitor of angiogenesis and growth of tumor metastases, is generated by cancer-mediated proteolysis of plasminogen. Human prostate carcinoma cells (PC-3) release enzymatic activity that converts plasminogen to angiostatin. We have now identified two components released by PC-3 cells, urokinase (uPA) and free sulfhydryl donors (FSDs), that are sufficient for angiostatin generation. Furthermore, in a defined cell-free system, plasminogen activators [uPA, tissue-type plasminogen activator (tPA), or streptokinase], in combination with one of a series of FSDs (*N*-acetyl-L-cysteine, D-penicillamine, captopril, L-cysteine, or reduced glutathione) generate angiostatin from plasminogen. An essential role of plasmin catalytic activity for angiostatin generation was identified by using recombinant mutant plasminogens as substrates. The wild-type recombinant plasminogen was converted to angiostatin in the setting of uPA/FSD; however, a plasminogen activation site mutant and a catalytically inactive mutant failed to generate angiostatin. Cell-free derived angiostatin inhibited angiogenesis *in vitro* and *in vivo* and suppressed the growth of Lewis lung carcinoma metastases. These findings define a direct mechanism for cancer-cell-mediated angiostatin generation and permit large-scale production of bioactive angiostatin for investigation and potential therapeutic application.

Because tumor growth and metastases are dependent upon angiogenesis (1–3), the identification of agents that inhibit angiogenesis now represents a potential therapeutic approach for the control of cancer (4–7). Angiostatin, consisting of the first four of five kringle domains of plasminogen (8), is one of a number of angiogenesis inhibitors that are internal fragments of larger nonangiogenic precursor proteins (8–14); however, the mechanisms by which these fragments are generated *in vivo* remains unknown. Although the activity sufficient to cleave plasminogen to angiostatin is present in tumor-bearing animals and serum-free conditioned medium (SFCM) of human prostate carcinoma cells (9), the cancer-dependent mechanism of angiostatin generation has remained unknown. Recently, macrophage-derived metalloelastase was shown to produce angiostatin from plasminogen and may contribute to angiostatin generation in the murine Lewis lung carcinoma model (15). We now describe the enzymatic mechanism for the direct generation of human angiostatin from plasminogen by human prostate cancer cells and demonstrate the generation of bio-

active angiostatin from human plasminogen in a defined cell-free system.

MATERIALS AND METHODS

Angiostatin Generation. Angiostatin was generated from PC-3 cell SFCM as described (9). To generate angiostatin in a cell-free system, human plasminogen (0.2 μ M) was incubated with 0.2 nM recombinant human urokinase (uPA; Abbott), 1.0 nM recombinant human two-chain tissue-type plasminogen activator (tPA; a gift from Henry Berger, Glaxo-Wellcome), or 8.0 nM streptokinase (Sigma) and with 100 μ M *N*-acetyl-L-cysteine (NAC), D-penicillamine, captopril, L-cysteine, or reduced glutathione (Sigma) at 37°C overnight. To confirm the requirement for plasmin catalytic activity, recombinant plasminogens (16) (0.2 μ M) were added to 100- μ l aliquots of 50 mM Tris, pH 9.0/20 mM NaCl/0.2 nM human recombinant uPA (Abbott)/100 μ M NAC (Sigma) and incubated at 37°C overnight. The angiostatin product was examined by Western blot as described (9).

Protein Purification. SFCM was applied to Reactive Red 120-agarose (Sigma) equilibrated with 50 mM Tris-HCl, pH 7.5/140 mM NaCl (TBS), and proteins were eluted with 1.0 M KCl. The eluate was dialyzed against TBS by using a 6- to 8-kDa cutoff membrane. Human plasminogen (0.2 μ M) was incubated in 100- μ l aliquots of SFCM, Reactive Red 120-agarose flow-through, dialyzed eluate, or combined flow-through and eluate at 37°C for 18 h. For anion-exchange chromatography, SFCM was diluted 1:5 in 50 mM Tris (pH 10.0) and applied to a High Q anion-exchange resin (Bio-Rad), and a linear gradient (50–300 mM NaCl/50 mM Tris, pH 10.0) was used for elution. The protein content of each fraction was estimated by measuring the absorbance at 280 nm and fractions were analyzed for angiostatin-generating activity. For isoelectric focusing, the SFCM was concentrated 10-fold by ultrafiltration (Amicon) using a molecular mass cutoff of 10 kDa and diluted 1:5 with sterile water to lower the NaCl concentration to 20 mM, and ampholyte carriers (pH 3.5–9.5, Bio-Rad) were added. The sample was then fractionated in a Rotofor Cell (Bio-Rad) that stabilized the proteins into 20 focused zones from pH 3.0 to 10.0. Each fraction was analyzed for pH and angiostatin-generating activity.

Cofactor Detection. Human plasminogen (0.2 μ M) was added to 100- μ l aliquots of the dialyzed Reactive Red 120-

Abbreviations: uPA, urokinase-type plasminogen activator; tPA, tissue-type plasminogen activator; NAC, *N*-acetyl-L-cysteine; bFGF, basic fibroblast growth factor; FSD, free sulfhydryl donor; SFCM, serum-free conditioned medium; AS_{CF}, angiostatin produced in the cell-free system.

††To whom reprint requests should be addressed at: Northwestern University, School of Medicine, 320 East Superior Street, Searle Building, 3–565, Chicago, IL 60611. e-mail: gasoff@merle.acns.nwu.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1997 by The National Academy of Sciences 0027-8424/97/9410868-5\$2.00/0 PNAS is available online at <http://www.pnas.org>.

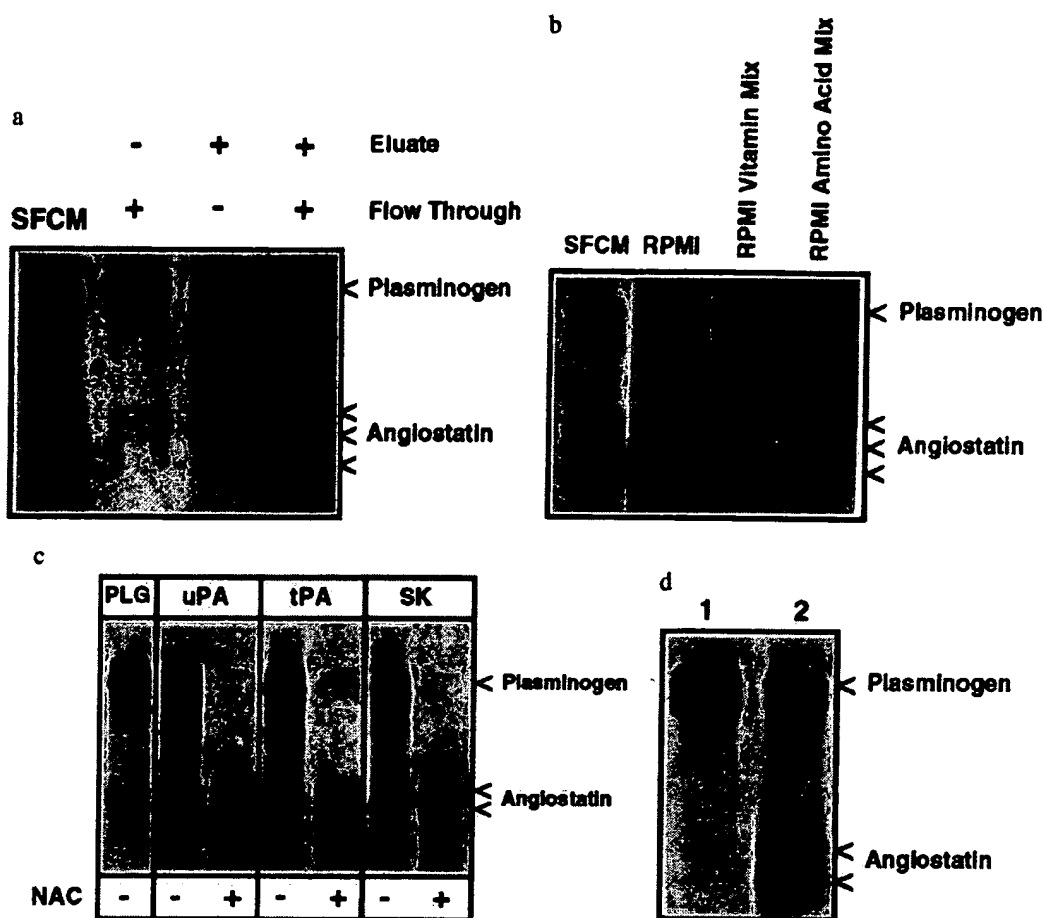


FIG. 1. Western blot analysis of angiotensin generation. (a) The angiotensin-generating activity of PC-3 SFCM required two distinct fractions from Reactive Red 120-agarose chromatography. Angiotensin was generated when plasminogen was incubated with SFCM. No angiotensin generation was detected by incubation of plasminogen with the Reactive Red 120-agarose flow-through or 1.0 M KCl eluate dialyzed against TBS. When the flow-through and dialyzed eluate were combined, however, angiotensin generation was restored. (b) A cofactor for angiotensin generation was present in unconditioned RPMI 1640 medium and its amino acid mixture. Plasminogen incubated with the dialyzed Reactive Red 120-agarose eluate supplemented with unconditioned RPMI 1640 medium also generates angiotensin. The cofactor activity of RPMI 1640 medium, necessary for angiotensin generation, was not present in the RPMI 1640 vitamin mixture but was present in the RPMI 1640 amino acid mixture. (c) Angiotensin was generated in a cell-free system consisting of human 0.2 μ M plasminogen (PLG), uPA, tPA, or streptokinase and the sulfhydryl donor *N*-acetyl-L-cysteine (NAC). Human plasminogen incubated with uPA (0.2 nM), tPA (1.0 nM), or streptokinase (8.0 nM) generated angiotensin only in the presence of NAC (100 μ M). (d) Human PC-3 prostate carcinoma cells, cultured for 24 h in the sulfhydryl-depleted RPMI 1640 medium, secreted sufficient FSDs and uPA to generate angiotensin from plasminogen. Human plasminogen was incubated with sulfhydryl-depleted RPMI 1640 medium and uPA (lane 1) or with identical sulfhydryl-depleted RPMI 1640 medium conditioned by human PC-3 prostate carcinoma cells (lane 2).

agarose eluate supplemented with components in RPMI 1640 medium: salts, phenol red, a vitamin mixture, or an amino acid mixture (GIBCO/BRL). To define the cofactor necessary for angiotensin generation, the dialyzed eluate was incubated with individual components of RPMI 1640 medium, and samples were tested for angiotensin-generating activity by Western blot.

Plasminogen Activator Detection. Fractions from the anion-exchange and Reactive Red 120-agarose eluates were examined with a coupled assay that measures plasminogen activation by monitoring the amidolytic activity of generated plasmin. Briefly, the eluates were dialyzed against TBS and incubated with plasminogen (0.3 μ M) and the plasmin substrate D-Val-Leu-Lys-p-nitroanilide (0.3 mM; Sigma) at 37°C. Substrate cleavage was determined by monitoring the absorbance at 405 nm using a kinetic plate reader (Molecular Devices).

Plasmin Generation. Human plasminogen (0.2 μ M) in 100- μ l aliquots of 50 mM Tris, pH 9.5/20 mM NaCl was incubated with 10 μ l of uPA-Sepharose (Calbiochem) for 2 h at 37°C. After incubation, the sample was centrifuged to sediment the uPA-Sepharose, and the supernatant containing

plasmin was collected. The complete conversion of plasminogen to plasmin was confirmed by analysis of the supernatant on reduced Coomassie-stained polyacrylamide gels. Plasmin was then incubated for 18 h with 100 μ M NAC, and samples were analyzed for the presence of angiotensin.

Bioactivity of Angiotensin. The angiotensin, generated in a cell-free system, was purified by affinity chromatography on lysine-Sepharose (Pharmacia Biotech) and examined on Western blots as described in Gately *et al.* (9). Endothelial cell migration assays were performed in a modified Boyden chamber with bovine adrenal capillary endothelial cells (a gift from J. Folkman) as described (17). The mouse corneal angiogenesis assays were performed as described (18).

The Lewis lung carcinoma metastasis model was performed as described by O'Reilly *et al.* (8). In brief, 1×10^6 low-metastatic Lewis lung carcinoma cells were inoculated subcutaneously into C57BL/6/J mice (The Jackson Laboratory). When tumors reached approximately 1200–1800 mg in size (12–14 days after implantation), animals were randomly divided into one of three treatment conditions: For the positive control group, mice were left with tumors intact ($n = 10$); for

the remaining animals, tumors were surgically resected. Tumor-resected mice received either cell-free-derived angiostatin, (0.15 mg, twice daily, subcutaneously) beginning on day 2 after surgery ($n = 6$) or, for negative control, received twice daily subcutaneous injections of phosphate-buffered saline ($n = 6$). Mice were sacrificed on days 25–27, and lung mass was measured to quantitate the growth of metastatic lung tumors.

RESULTS

Purification of the Factors Responsible for the Production of Angiostatin. A significant loss of angiostatin-generating activity from human PC-3 prostate carcinoma cell SFCM (9) was observed after dialysis using 6- to 8-kDa molecular mass cutoff membranes, suggesting that a low molecular weight cofactor was required. Fractionation of SFCM on Reactive Red 120-agarose indicated that complementary components were required for angiostatin generation. The flow-through and the dialyzed eluate alone failed to generate angiostatin; however, combination of the flow-through and the eluate fraction restored angiostatin-generating activity (Fig. 1*a*). The flow-through component was stable to boiling, suggesting that this factor was not likely to be a protein. In contrast, the eluate component was thermolabile and was retained after dialysis consistent with the eluate containing a protein(s).

The Flow-Through Component Necessary for Angiostatin Generation Was Identified as a Free Sulfhydryl Donor (FSD). Addition of nonconditioned RPMI 1640 medium to the Reactive Red 120-agarose eluate resulted in the generation of angiostatin (Fig. 1*b*), indicating a component of RPMI 1640 medium could serve as a cofactor. Individual constituents of nonconditioned RPMI 1640 medium were then incubated with the dialyzed eluate. The amino acid mixture could complement the eluate for angiostatin generation (Fig. 1*b*), and testing of individual amino acids at concentrations present in RPMI 1640 medium indicated that L-cysteine is the only amino acid capable of complementing the eluate. The RPMI vitamin mixture (Fig. 1*b*) and other RPMI constituents could not serve as a cofactor. Because L-cysteine is a FSD, reduced glutathione (100 μ M) and NAC (100 μ M) were evaluated and also found to effectively complement the eluate for angiostatin generation.

The Protein in the Elution Necessary for Angiostatin Generation Is a Plasminogen Activator. Fractionation of SFCM using isoelectric focusing indicated that angiostatin generation was associated with an isoelectric point of approximately 9.2, similar to uPA (19). Furthermore, anion-exchange chromatography of SFCM resulted in the copurification of the angiostatin-generating activity with uPA, and the Reactive Red 120-agarose eluate contained plasminogen activator activity. The inability to separate uPA from angiostatin-generating activity suggested a role for uPA in angiostatin generation. The observation that plasmin was also converted to angiostatin by PC-3 SFCM (9) suggested that prior conversion of plasminogen to plasmin would not be inhibitory for angiostatin generation. Human plasminogen was therefore incubated with catalytic amounts of uPA, tPA, and streptokinase with and without NAC (100 μ M; Fig. 1*c*). These data demonstrate that a plasminogen activator and NAC were sufficient for the complete conversion of plasminogen to angiostatin. Additional experiments indicated that other FSDs (100 μ M L-cysteine, 100 μ M reduced glutathione, 100 μ M D-penicillamine, or 100 μ M captopril) could substitute for NAC for the production of angiostatin. Incubation of plasminogen with uPA and a nonsulfhydryl reducing agent, dextrazoxane (Zincard, Pharmacia), did not generate angiostatin, demonstrating the specific requirement for a FSD. These data indicate that incubation of human plasminogen with a plasminogen activator and a FSD is sufficient for conversion to angiostatin.

Prostate Carcinoma Cells Release FSDs *in Vitro*. Because RPMI 1640 medium contains FSDs in the form of L-cysteine and glutathione, to determine whether PC-3 cells release sufficient FSD to convert plasminogen/plasmin to angiostatin, PC-3 cells were cultured for 24 h in defined RPMI 1640 medium lacking reduced glutathione, L-cysteine, and L-methionine. This PC-3 SFCM was found to efficiently catalyze the conversion of plasminogen to angiostatin, indicating the cells release sufficient FSD and uPA for angiostatin generation (Fig. 1*d*).

Plasmin as a Substrate for Angiostatin Generation. Purified human plasmin, in the absence of uPA or other plasminogen activators, is converted to angiostatin in the presence of a FSD, suggesting a direct effect of the sulfhydryl donor on plasmin enzymatic activity or substrate specificity (Fig. 2*a*). To confirm a role for plasminogen conversion to plasmin and the catalytic role of plasmin in angiostatin generation, recombinant plasminogens (16) were evaluated as substrates for angiostatin generation. The R561A plasminogen activation site mutant is not susceptible to cleavage by plasminogen activators, whereas the D646E mutant yields a catalytically inactive plasmin due to a substitution of an essential amino acid in the serine proteinase catalytic domain. Both plasma-derived plasminogen and the wild-type recombinant plasminogen were converted to angiostatin when incubated with uPA and 100 μ M NAC (Fig.

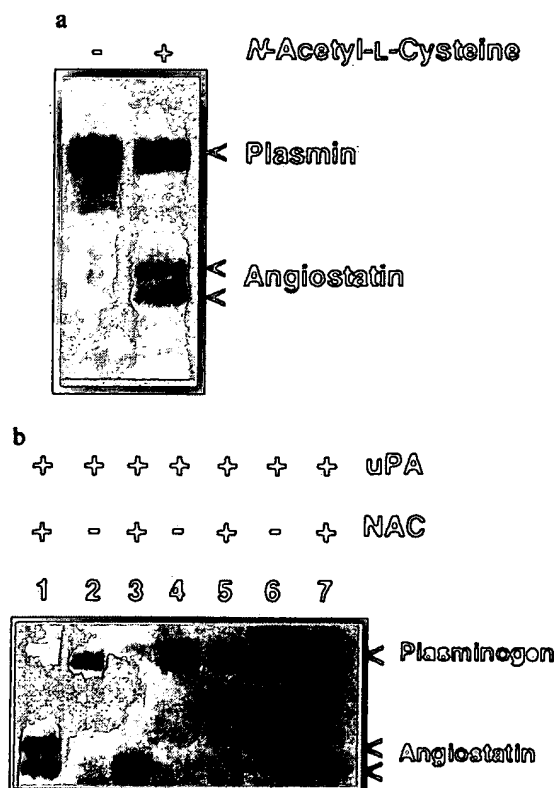


Fig. 2. (a) Plasmin is converted to angiostatin in the presence of a FSD. Human plasminogen was converted to plasmin by incubation with uPA-Sepharose. Plasmin was only converted to angiostatin in the presence of 100 μ M NAC. (b) Plasmin generation and catalytic activity is essential for angiostatin generation. Plasma-derived human plasminogen (0.2 μ M), incubated with uPA (0.2 nM) and NAC (NAC) generates angiostatin (lane 1). The recombinant wild-type plasminogen (lanes 2 and 3) is also converted to angiostatin by the addition of uPA and NAC. The R561A activation site mutant (lanes 4 and 5), not susceptible to activation by plasminogen activators, failed to generate angiostatin when incubated with uPA and NAC. The D646E catalytically inactive mutant (lanes 6 and 7) also failed to generate angiostatin, demonstrating the requirement for plasmin catalytic activity.

2b). However, the R561A mutant was not cleaved to plasmin or angiostatin under these conditions, providing further evidence that plasmin is an essential intermediate in angiostatin generation. The D646E mutant was converted to two-chain plasmin but angiostatin was not generated, demonstrating that plasmin catalytic activity is necessary for angiostatin generation (Fig. 2b).

Bioactivity of Affinity-Purified Cell-Free-Derived Angiostatin. The affinity-purified angiostatin produced in the cell-free system (AS_{CF}) was biologically active, suppressing basic fibroblast growth factor (bFGF)-induced endothelial cell proliferation with an ED₅₀ of approximately 15 μ g/ml, similar to the PC-3-derived angiostatin (9). Inhibition of bFGF-induced endothelial cell migration *in vitro* by the cell-free angiostatin was comparable to the PC-3-derived (9) and elastase-generated angiostatin (generously provided by Michael O'Reilly, Harvard Medical School), with an observed ED₅₀ of 0.33 μ g/ml (Fig. 3). As shown in Table 1, the cell-free angiostatin inhibited bFGF-induced angiogenesis in the mouse cornea as was shown for the PC-3-derived angiostatin (9).

Administration of the cell-free-produced angiostatin to mice significantly inhibited the growth of Lewis lung carcinoma metastases (Fig. 4). Surgical resection of primary subcutaneous Lewis lung tumors in mice resulted in numerous macroscopic metastases and a 71% increase in lung mass compared with animals in which the primary tumors were not resected (Fig. 4). By contrast, administration of angiostatin produced in a cell-free system suppressed the increase in lung weight to a comparable level as observed in animals with the primary tumor intact, and only microscopic metastases were observed. These data not only support the model that primary tumors can suppress the growth of metastases by the generation of an inhibitor of angiogenesis, angiostatin, but also confirm the biological activity of the cell-free-produced angiostatin.

DISCUSSION

The results presented demonstrate the mechanism by which human prostate carcinoma cells convert plasminogen to the

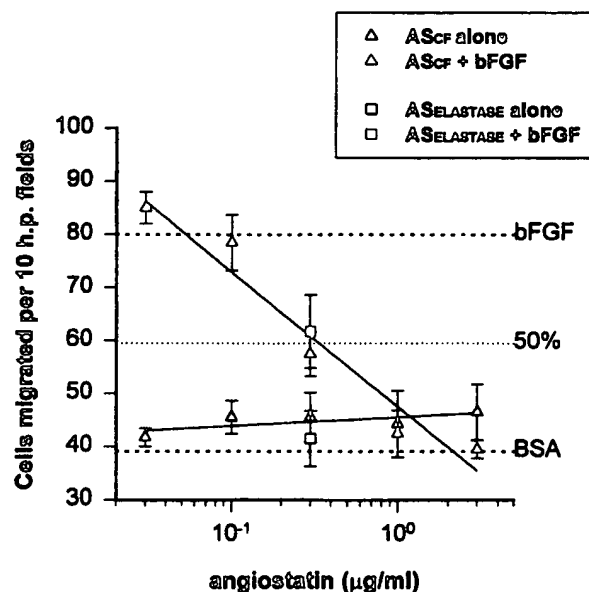


FIG. 3. Inhibition of bovine capillary endothelial cell migration by angiostatin produced in a cell-free system (AS_{CF}). Endothelial cell migration in a Boyden chamber toward a range of concentrations of angiostatin was measured in the presence (solid symbol) or absence (open symbol) of stimulatory bFGF. As a control, single points using elastase-generated angiostatin (AS_{Elastase}) are shown (squares). These data demonstrate that AS_{CF} inhibits bFGF-induced endothelial cell migration in a dose-dependent manner, with an ED₅₀ of 0.33 μ g/ml.

Table 1. *In vivo* inhibitory activity of cell-free produced angiostatin

| Compound tested | No. positive corneas/ total no. implanted |
|---------------------------------|--|
| bFGF (50 ng per pellet) | 4/4 |
| Angiostatin (200 ng per pellet) | 0/4 |
| bFGF + angiostatin | 0/4 |

Pellets were formulated with the indicated compounds and implanted into the corneas of mice, and neovascularization was assessed by slit-lamp microscopy 5 days later. Vigorous growth of vessels into the normally avascular cornea was scored as a positive response.

angiogenesis inhibitor angiostatin. Plasminogen is first converted to the two-chain serine proteinase plasmin, by uPA, and in the presence of a FSD, plasmin serves as both the substrate and enzyme for the generation of angiostatin (Fig. 5). This pathway was confirmed by the ability to convert plasminogen to angiostatin in a cell-free system using one of three available plasminogen activators and one of a series of physiological or pharmacological FSDs. Furthermore, the angiostatin generated in the cell-free system was shown to be bioactive, demonstrating antiangiogenic activity *in vitro* and *in vivo* and suppressing the growth of lung metastases in the mouse Lewis lung carcinoma model.

The local or systemic availability of FSDs may be an important regulatory point in the angiogenic cascade in physiologic and pathologic settings. The role of the FSD is not yet known but could be involved in modification of the conformation of plasmin, altering enzymatic activity or allowing plasmin to be cleaved at previously unrecognized sites. The observation that a FSD is required for angiostatin generation suggests that the reported antiangiogenic properties of pharmacologic sulfhydryl donors such as D-penicillamine and captopril (20–24) may be due to their ability to promote the conversion plasmin, a normally proangiogenic proteinase (25), to the angiogenic inhibitor angiostatin. The potential loss of plasmin catalytic activity that would result from plasmin conversion to angiostatin (Fig. 5) may contribute to reduced

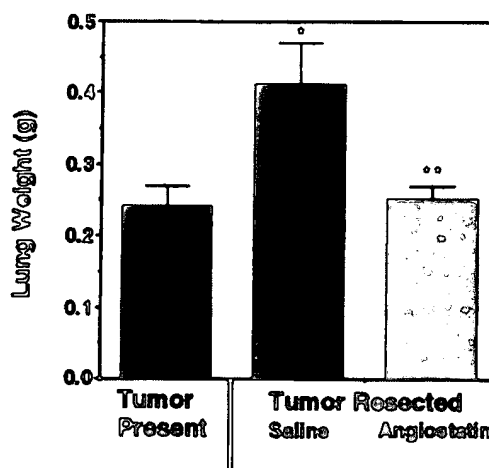


FIG. 4. Angiostatin produced in a cell-free system suppresses the growth of Lewis lung carcinoma lung metastases after resection of the primary tumor. The presence of the primary subcutaneous Lewis lung tumor suppressed the expansion of lung metastases (tumor present control). By contrast resection of the Lewis lung tumor and administration of saline resulted in a significant increase in the mean lung mass compared with the tumor present control, confirming primary tumor-mediated suppression of metastatic tumor growth (*, $P < 0.01$). Subcutaneous administration of angiostatin after removal of the primary tumor, significantly suppressed the expansion of lung metastases to levels comparable to the tumor control group (angiostatin compared with saline; **, $P < 0.01$).

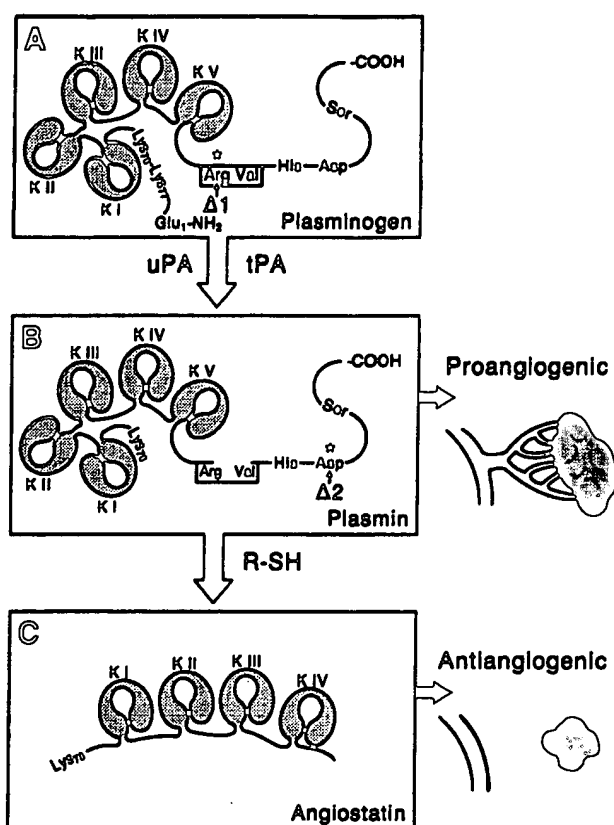


FIG. 5. Conversion of the proangiogenic proteinase plasmin to the angiogenesis inhibitor angiostatin. (A) The zymogen plasminogen is converted to the active proteinase plasmin by cleavage of the Arg⁵⁶⁰-Val⁵⁶¹ peptide bond by plasminogen activators such as uPA and tPA. (B) Plasmin is a proangiogenic proteinase capable of degrading a variety of extracellular matrix proteins, facilitating endothelial cell migration and angiogenesis. (C) Plasmin in the presence of a FSD is converted to the angiogenesis inhibitor angiostatin. The plasminogen activation site mutant R561A, indicated by $\Delta 1$, is not cleaved by plasminogen activators, preventing conversion of plasminogen to the plasmin intermediate required for angiostatin generation. The plasminogen mutant D646E, indicated by $\Delta 2$, is cleaved by plasminogen activators, but the resulting two-chain plasmin is inactive due to the substitution of a catalytically essential aspartic acid residue in the serine proteinase catalytic triad. In the presence of a FSD, the inactive D646E mutant plasmin is not converted to angiostatin, demonstrating the requirement for plasmin catalytic activity.

fibrinolysis and the hypercoagulable state often observed in patients with cancer (26).

The angiostatin-generating activity released by human prostate carcinoma cells was not blocked by inhibitors of elastase or metal-dependent proteinases (9). These data suggest a direct mechanism of angiostatin generation by human prostate cancer cells, in contrast to the indirect mechanism of angiostatin generation, dependent upon expression of metalloelastase by tumor-infiltrating macrophages (15). Thus, these data demonstrate alternative pathways for the generation of angiostatin.

The identification of a direct mechanism of human prostate cancer-mediated angiostatin generation and the recapitulation of this process in a cell-free system allow for the efficient large-scale production of angiostatin that is antiangiogenic and capable of suppressing the growth of Lewis lung carcinoma metastases. The ability to produce angiostatin in a cell-free

system will allow for large-scale production of this protein for *in vivo* testing as a novel anticancer agent. In addition, the elucidation of the components required for plasminogen conversion to angiostatin could permit the direct *in vivo* generation of angiostatin in the patient by administration of a plasminogen activator with a pharmacologic FSD.

We thank M.S. O'Reilly for providing the elastase-generated angiostatin and R. Bacallao for assistance with isoelectric focusing. This work was supported in part by The Feinberg Cardiovascular Research Institute; a grant-in-aid from the American Cancer Society, Illinois Division; National Institutes of Health Grants CA71875 (to G.A.S.), CA58900 (to M.S.S.), and HL13423 (to F.J.C.); and Veterans Administration Merit Review Research Grant (to H.C.K.).

1. Folkman, J. (1990) *J. Natl. Cancer Inst.* **82**, 4-6.
2. Folkman, J., Watson, K., Ingber, D. & Hanahan, D. (1989) *Nature (London)* **339**, 58-61.
3. Weidner, N., Semple, J. P., Welch, W. R. & Folkman, J. (1991) *N. Engl. J. Med.* **324**, 1-8.
4. Fotis, T., Zhang, Y., Pepper, M. S., Adlercreutz, H., Montesano, R., Nawroth, P. P. & Schweigerer, L. (1994) *Nature (London)* **368**, 237-239.
5. Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S. & Ferrara, N. (1993) *Nature (London)* **362**, 841-844.
6. O'Reilly, M. S., Holmgren, L., Chen, C. & Folkman, J. (1996) *Nat. Med.* **2**, 689-692.
7. Holmgren, L., O'Reilly, M. S. & Folkman, J. (1995) *Nat. Med.* **1**, 149-153.
8. O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H. & Folkman, J. (1994) *Cell* **79**, 315-328.
9. Gately, S., Twardowski, P., Stack, M. S., Patrick, M., Boggio, L., Cundiff, D. L., Schnaper, H. W., Madison, L., Volpert, O., Bouck, N., Enghild, J., Kwaan, H. C. & Soff, G. A. (1996) *Cancer Res.* **56**, 4887-4890.
10. O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R. & Folkman, J. (1997) *Cell* **88**, 277-285.
11. Gupta, S. K., Hassel, T. & Singh, J. P. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 7799-7803.
12. Sage, E. H., Bassuk, J. A., Yost, J. C., Folkman, M. J. & Lane, T. F. (1995) *J. Cell. Biochem.* **57**, 127-140.
13. Clapp, C., Martial, J. A., Guzman, R. C., Rentier-Delure, F. & Weiner, R. I. (1993) *Endocrinology* **133**, 1292-1299.
14. Good, D. J., Polverini, P. J., Rastinejad, F., Le Beau, M. M., Lemons, R. S., Frazier, W. A. & Bouck, N. P. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 6624-6628.
15. Dong, Z., Kumar, R., Yang, X. & Fidler, I. J. (1997) *Cell* **88**, 801-810.
16. Grella, D. & Castellino, F. J. (1997) *Blood* **89**, 1585-1589.
17. Rastinejad, F., Polverini, P. J. & Bouck, N. P. (1989) *Cell* **56**, 345-355.
18. Kenyon, B. M., Voest, E. E., Chen, C. C., Flynn, E., Folkman, J. & D'Amato, R. J. (1996) *Invest. Ophthalmol. Visual Sci.* **37**, 1625-1632.
19. Colombi, M., Rebessi, L., Boiocchi, M. & Barlati, S. (1986) *Cancer Res.* **46**, 5748-5753.
20. Volpert, O. V., Ward, W. F., Ling, M. W., Chesler, L., Solt, D. B., Johnson, M. D., Molteni, A., Polverini, P. J. & Bouck, N. P. (1996) *J. Clin. Invest.* **98**, 671-679.
21. Brem, S. S., Zagzag, D., Tsanacis, A. M., Gately, S., Elkouby, M. P. & Brien, S. E. (1990) *Am. J. Pathol.* **137**, 1121-1142.
22. Matsubara, T., Saura, R., Hirohata, K. & Ziff, M. (1989) *J. Clin. Invest.* **83**, 158-167.
23. Matsubara, T. & Ziff, M. (1987) *J. Clin. Invest.* **79**, 1440-1446.
24. Koch, A. E., Burrows, J. C., Polverini, P. J., Cho, M. & Leibovich, S. J. (1991) *Agents Actions* **34**, 350-357.
25. Mignatti, P. & Rifkin, D. B. (1996) *Curr. Top. Microbiol. Immunol.* **213**, 33-50.
26. Green, K. B. & Silverstein, R. L. (1996) *Hematol. Oncol. Clin. N. Am.* **10**, 499-530.

Human Angiostatin Inhibits Murine Hemangioendothelioma Tumor Growth *in Vivo*¹Brian J. Lannutti, Stephen T. Gately, M. Eugenia Quevedo, Gerald A. Soff, and Amy S. Paller²

Departments of Pediatrics [B. J. L., M. E. Q., A. S. P.], Dermatology [A. S. P.], and Medicine, Division of Hematology/Oncology [S. T. G., G. A. S.], Northwestern University Medical School, Chicago, Illinois 60611

Abstract

Angiostatin inhibits angiogenesis and metastatic tumor growth; however, its usefulness in treating primary nonmetastasizing tumors is less well understood. We now report the effectiveness of human angiostatin administration in a mouse hemangioendothelioma model. Human angiostatin was administered to mice with s.c. hemangioendothelioma and associated disseminated intravascular coagulopathy (Kasabach-Merritt syndrome). Angiostatin significantly reduced tumor volume in comparison to nontreated controls, increased survival, and prevented the profound thrombocytopenia and anemia of Kasabach-Merritt syndrome. Apoptosis of tumor cells was induced by angiostatin, but tumor cell proliferation was not inhibited. These data suggest angiostatin as a novel treatment for nonmetastasizing vascular tumors and for Kasabach-Merritt syndrome.

Introduction

Solid tumor growth and metastases are dependent on angiogenesis (1). Angiostatin is a potent, endogenous inhibitor of angiogenesis that is generated by the proteolytic cleavage of plasminogen (2-4). Angiostatin has been shown to inhibit the growth of metastatic tumors (2, 5-8); however, its role in the management of primary tumors that do not metastasize has not been explored. The Kasabach-Merritt syndrome is a life-threatening condition in infants that is characterized by rapid enlargement of a primary endothelial cell-derived tumor in association with disseminated intravascular coagulopathy (9). Using a mouse model of the Kasabach-Merritt syndrome (10), we have tested the ability of affinity-purified angiostatin to control primary hemangioendothelioma growth. We now report that human angiostatin markedly suppressed the growth of s.c. hemangioendothelioma, prevented the hematological complications of Kasabach-Merritt, and increased mouse survival.

Materials and Methods

Cell Culture. The murine hemangioendothelioma cell line EOMA (generously provided by Dr. R. Auerbach, University of Wisconsin, Madison, WI) and primary bovine aortic endothelial cells isolated from calf aortas (11) were grown in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 mg/ml streptomycin (Life Technologies, Inc., Gaithersburg, MD) and maintained at 37°C.

Angiostatin Generation. Affinity-purified human angiostatin was generated in a cell-free setting as described previously (3). Briefly, human plasminogen (0.2 μ M) was incubated with 0.2 nM recombinant human urokinase

(Abbott Laboratories, North Chicago, IL) and 100 μ M of *N*-acetyl-L-cysteine (Sigma Chemical Co.) at 37°C overnight. The angiostatin was then affinity purified on lysine-Sepharose and examined by Western blot assays as described previously (12). Each lot of angiostatin was tested for the ability to inhibit bFGF³-induced bovine aortic endothelial cell proliferation *in vitro*, prior to administration *in vivo*. In addition, we have shown that the angiostatin generated by recombinant human urokinase/*N*-acetyl-L-cysteine treatment inhibits normal endothelial cell migration *in vitro* and corneal vessel angiogenesis *in vivo* (3).

Bioactivity of Angiostatin *in Vitro*. To assess the antiproliferative effects of angiostatin, bovine aortic endothelial and EOMA cells were plated at 2.0×10^4 cells/well in 24-well culture plates and grown overnight. Medium was then replaced with fresh DMEM/5% heat-inactivated fetal bovine serum supplemented with 1 ng/ml bFGF (R & D Systems, Minneapolis, MN) with and without affinity-purified human angiostatin (50, 100, or 200 nM). Cell counts from duplicate wells were performed after 48 h incubation using a ZBI Coulter counter (Coulter Corp., Hialeah, FL).

***In Vitro* Apoptosis Assay.** To determine whether angiostatin induces apoptosis of EOMA cells *in vitro*, 1.0×10^4 EOMA cells were plated on four-chambered slides (Lab-Tec; Nalgene Nunc International, Naperville, IL) in duplicate in serum-containing medium. Sixteen h later, cells were incubated in either serum-containing or serum-free DMEM with or without angiostatin (0-200 nM) for 24 h. Cells were washed, and DNA fragmentation was detected using a Klenow FragEL DNA fragmentation kit (Oncogene, Cambridge, MA). The binding of biotinylated nucleotides was detected by streptavidin-horseradish peroxidase, followed by hydrogen peroxide/diaminobenzidine and counterstaining with methyl green. For a positive control, cells were treated with proteinase K, and then 1.0 μ g/ml DNase I in TBS containing 1.0 nM MgSO₄ was applied. For a negative control, TBS was substituted for Klenow.

Western Blot. Western blots were performed as described previously (12). Circulating angiostatin was detected in mice treated with angiostatin ($n = 4$) and PBS ($n = 4$). Plasma protein concentrations were measured by a modified Bradford assay (Ref. 13; Coomassie Plus Protein Assay; Pierce, Rockford, IL). The mouse plasma samples were standardized, diluted 1:20 with TBS for a final loading concentration of 250 ng/lane, electrophoresed under nonreducing conditions on 12% polyacrylamide gels (NOVEX, San Diego, CA) in Tris-Glycine running buffer, and electrotransferred to a 0.45 μ M polyvinylene difluoride membrane (Immobilon; Millipore, Bedford, MA). Purified human angiostatin 1 μ g/lane was run as a control. The membrane was blocked for 30 min in 1% BSA in TBS and probed with a 1:1000 dilution of a monoclonal antibody to the kringle 1-3 domains of human plasminogen (VAP 250 L; Enzyme Research Laboratories, Inc., South Bend, IN). After washing, the membrane was incubated for 30 min with an alkaline phosphatase conjugated goat anti-mouse IgG secondary antibody and developed using 5-bromo-4-chloro-3-indoyl-phosphate/nitroblue tetrazolium (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Murine Tumor Model. Animal experiments were approved by the Institutional Animal Care and Use Committee of the Children's Memorial Institute for Education and Research. Female beige nude mice (Taconic Labs, Germantown, NY) at 6-8 weeks of age received injections s.c. in the right flank with 1.0×10^6 EOMA cells in 100 μ l PBS. Tumors appeared approximately 7 days after implantation and grew exponentially until the mice died. Beginning 24 h

Received 8/28/97; accepted 10/17/97.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by March of Dimes Grant 96-0455 (to A. S. P.), grants from the Dermatology Foundation Dermik (to A. S. P.) and Feinberg Cardiovascular Research Institute, a grant-in-aid from the American Cancer Society, Illinois Division, and NIH Grant CA71875 (to G. A. S.).

² To whom requests for reprints should be addressed, at Division of Dermatology #107, Children's Memorial Hospital, 2300 Children's Plaza, Chicago, IL 60614. Phone: (773) 880-3681; Fax: (773) 880-3025; E-mail: apaller@nwu.edu.

³ The abbreviations used are: bFGF, basic fibroblast growth factor; TBS, Tris-buffered saline; PCNA, proliferating cell nuclear antigen; KMS, Kasabach-Merritt syndrome.

after tumor cell inoculation, human angiotatin 40 mg/kg/dose, or the PBS vehicle, in a final volume of 400 μ l, was injected s.c. into the nuchal region of the mice twice daily until sacrifice. Mice were weighed every other day, and the tumors were measured three times weekly using tissue calipers. Tumor volume was determined using the formula $(\text{width})^2 \times \text{length} \times 0.52$ (5). Control mice were euthanized when tumors reached a diameter of 1.5–2.0 cm, when difficulty with ambulation and lethargy occurred. Mice treated with angiotatin were euthanized when the last control mouse was euthanized. Cardiac puncture was performed preterminally under deep anesthesia for collection of blood, then spleens and tumors were collected at autopsy and weighed. This experiment was performed in duplicate, with at least eight mice studied for each treatment condition.

Histological Studies. Tumor and spleen tissues were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and then stained with H&E for routine histological examination. To determine the percentage of apoptotic tumor cells, 5.0- μ m tissue sections were deparaffinized and washed. DNA fragmentation was detected as described for *in vitro* assays. To quantitate cell proliferation, tumor sections were immunostained with a monoclonal anti-PCNA antibody. Briefly, tissue sections were deparaffinized and treated with 0.05% trypsin (Sigma). After quenching with H_2O_2 and blocking with horse serum, samples were incubated with a 1:75 dilution of mouse monoclonal anti-PCNA antibody (DAKO Corp., Carpinteria, CA). Binding was detected by treatment with horse anti-mouse IgG using a Vectastain kit (Vector Laboratories, Burlingame, CA) and colorimetric staining with diaminobenzidine. The percentage of proliferative and apoptotic tumor cells were determined by counting a minimum of 4000 cells from 10 nonoverlapping microscope fields of tumor sections from at least four mice from each treatment condition under a light microscope at $\times 200$.

Determination of Platelet Levels and Hematocrits. Cell counts were determined using a CellDyn Coulter counter (Abbott Laboratories, Abbott Park, IL). Age-matched beige nude mice without EOMA tumors ($n = 4$) were used to determine the normal range of values.

Statistical Analysis. Results are expressed as mean \pm SD. Statistically significant differences between means were determined using a one-way ANOVA. A value of $P < 0.05$ was considered significant.

Results and Discussion

Angiotatin Inhibits Hemangioendothelioma Growth *in Vivo* but not *in Vitro*. Previous studies with angiotatin have focused on the ability of this potent antiangiogenic agent to inhibit the growth of metastases (2–4, 6) and, more recently, of metastasizing primary tumors (5–7). We have now tested the ability of affinity-purified human angiotatin to inhibit the growth of a primary, nonmetastasizing endothelial-derived tumor *in vivo*. Angiotatin significantly inhibited the growth of the murine hemangioendothelioma *in vivo*. No difference was noted in tumor volumes between treated and untreated mice until 10 days after tumor inoculation (Fig. 1A). However, by 17 days after tumor cell inoculation, the tumor volume of angiotatin treated mice was $66 \pm 12 \text{ mm}^3$ (Fig. 1B), whereas the tumor volume of control mice was $825 \pm 67 \text{ mm}^3$ (Fig. 1C). The wet weight of tumors in control-treated mice was also significantly higher than that of angiotatin-treated mice ($P < 0.001$; Fig. 2A). Angiotatin administration *in vitro* did not inhibit the proliferation of EOMA cells, at concentrations as high as 200 nM, despite the endothelial cell origin of the EOMA cells.

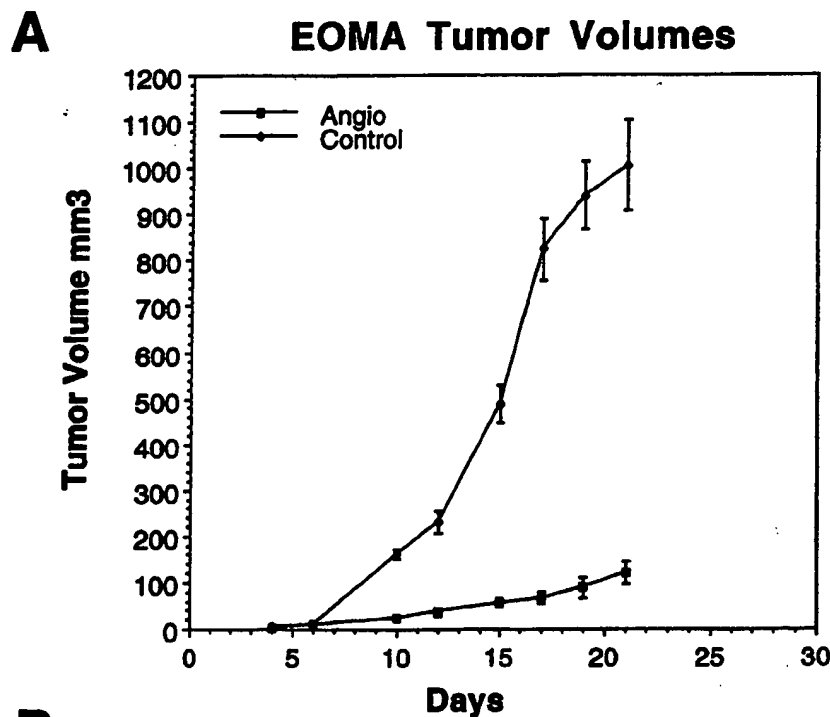


Fig. 1. A, tumor volumes of PBS- and angiotatin-treated animals. No statistically significant difference in the tumor volume was observed between PBS- and angiotatin-treated mice until 3 days after tumor appearance. By 10 days after tumor appearance, the mean volume of tumors in angiotatin treated mice (B) was significantly less than the volume of the control, PBS-treated mice (C; $P < 0.0001$).

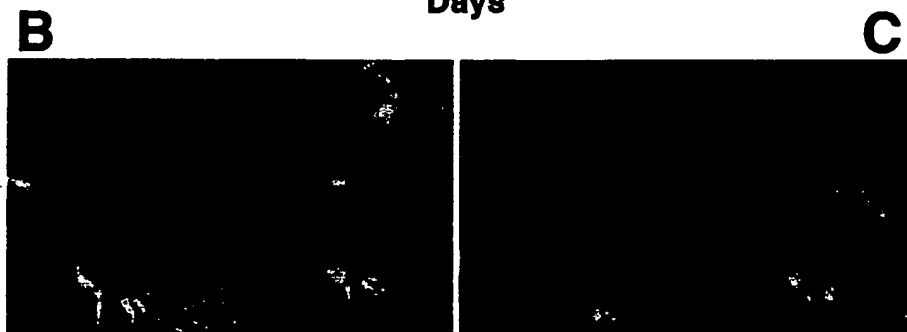
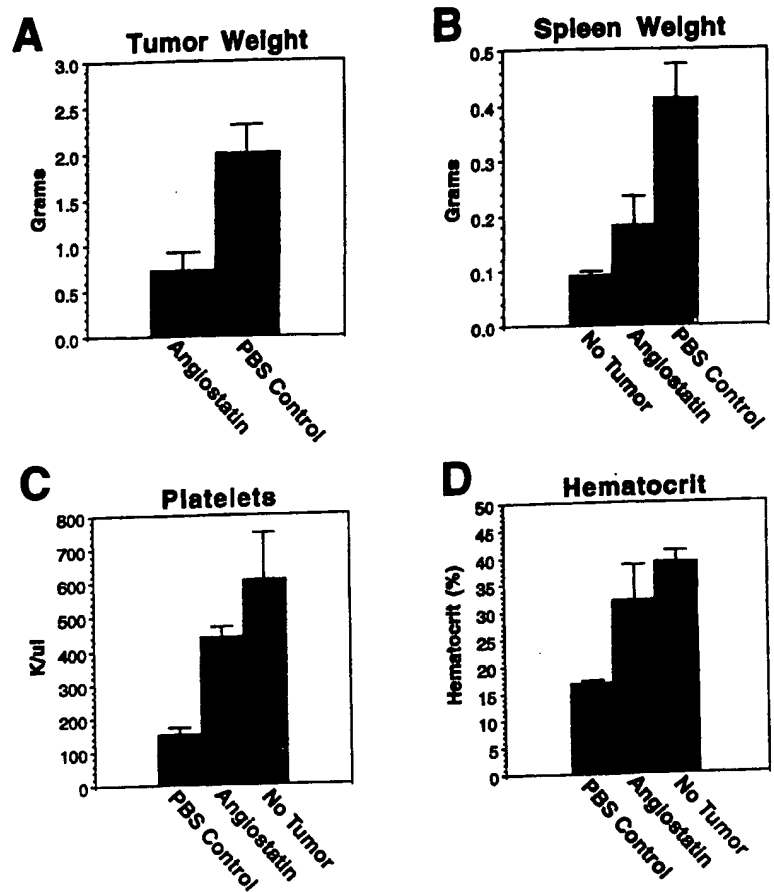


Fig. 2. Effect of angiostatin treatment on tumor weight, spleen weight, platelet counts, and hematocrit values. Control mice were euthanized between 15 and 20 days after inoculation when preterminal, and angiostatin-treated mice were euthanized at 20 days after inoculation. Tumors and spleens were weighed, and blood was taken by cardiac puncture for hematological studies. In A, the wet weight of tumors in control-treated mice was significantly higher than that of angiostatin-treated mice; in B, the spleen weight of angiostatin-treated mice was not significantly higher than age-matched control beige nude mice without hemangioendothelioma. By contrast, the spleen weight of control, PBS-treated mice with hemangioendothelioma was significantly higher than non-tumor-bearing control mice; in C, the platelet count in mice treated with angiostatin was not significantly lower than non-tumor-bearing control mice but was significantly lower in the control, PBS-treated mice; in D, angiostatin maintained the hematocrit levels, almost to the levels in normal mice without the tumor. In comparison, hematocrit levels in PBS-treated control mice were significantly reduced.



By contrast, bFGF-induced proliferation of nontransformed bovine endothelial cells was completely inhibited at 100 nM (data not shown). Angiostatin treatment also did not delay or prevent the appearance of tumors, evident at 6–7 days after inoculation (Fig. 1A); however, a statistically significant difference in tumor volume was evident by 10 days after tumor cell injection ($P < 0.0001$). These data suggest that the mechanism for this significant growth inhibition involves suppression of angiogenesis (5, 14, 15) rather than a primary effect on the endothelial cell-derived tumor cells themselves.

Untreated control mice had a mean age of death at 17 ± 3 days after tumor cell inoculation because of a large tumor burden, anemia, and internal bleeding related to the splenomegaly and decreased platelet counts (Fig. 2). At the time of death of the control mice, the angiostatin-treated mice showed no evidence of the pallor, lethargy, or bleeding into the skin that was observed in the control mice. The lack of apparent toxicity in our trials with 80 mg/kg/day angiostatin is consistent with previous studies in which no side effects were observed in mice that received as much as 100 mg/kg/day of elastase-generated angiostatin (5) or recombinant angiostatin (6, 7) for up to 60 days (5).

Angiostatin Prevents KMS. KMS is a life-threatening complication of endothelial cell tumors in infancy. The disorder is characterized by rapid enlargement of a tumor, usually a hemangioendothelioma, in association with thrombocytopenia, microangiopathic anemia, and coagulopathy (9, 16). Relatively weak inhibitors of angiogenesis, such as systemic corticosteroids and IFN- α (17, 18), have been administered. Despite aggressive intervention with these systemic medications, surgery, and anticoagulants, 20–50% of affected infants die, often because of the bleeding diathesis. s.c. injection of hemangioendothelioma (EOMA) cells provides a model of KMS, with mice developing progressive splenomegaly, thrombocytopenia, and microangiopathic anemia (10). AGM 1470, a

synthetic analogue of the fungal product fumagillin and a potent inhibitor of angiogenesis, has recently been shown to inhibit hemangioendothelioma growth (19), although its effect on the hematological complications of hemangioendothelioma growth was not studied. Using this model, we investigated the effect on hematological complications as well as tumor growth of angiostatin.

Angiostatin administration prevented the splenomegaly and hematological complications in tumor-bearing animals. The spleen weight

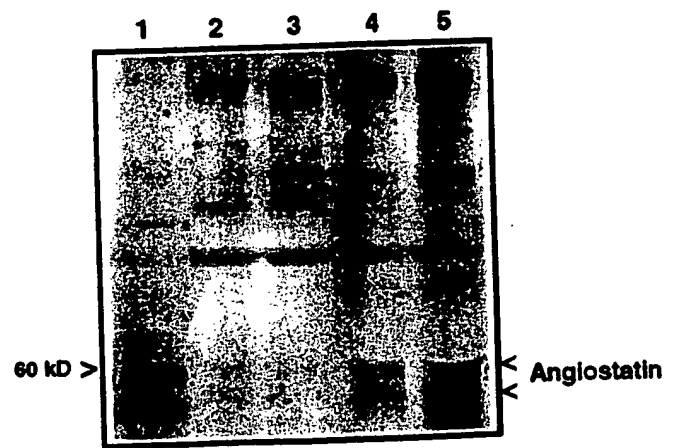


Fig. 3. Detection of injected angiostatin in mouse plasma. Mice were sacrificed, and blood was taken by cardiac puncture 24 h after the last injection of angiostatin. Affinity purified angiostatin shows a doublet at approximately 50 kD (Lane 1). Examination of plasma from PBS-treated mice reveals no detectable angiostatin (Lanes 2 and 3). By contrast, the plasma from mice treated with angiostatin demonstrates detectable angiostatin of similar size to the administered human angiostatin (Lanes 4 and 5).

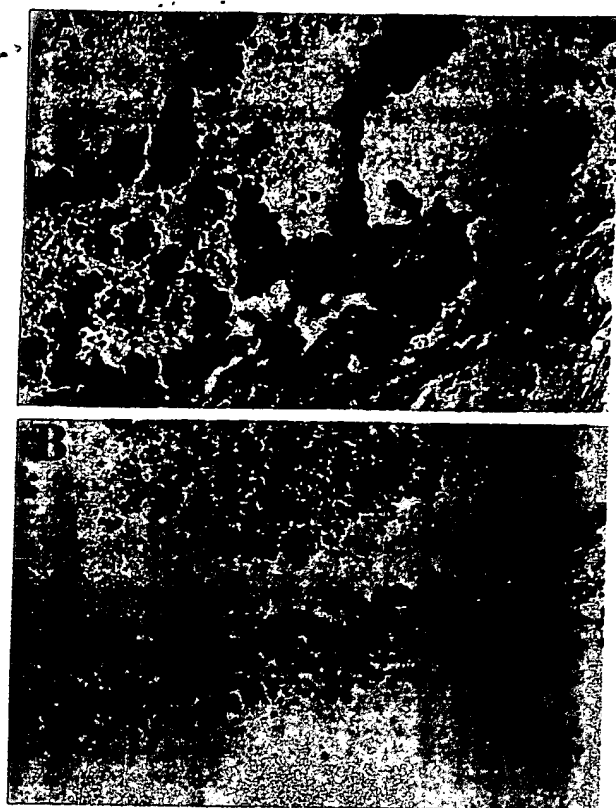


Fig. 4. Detection of apoptosis *in vivo*. Immunohistochemical analysis of sections of hemangioendothelioma tumors demonstrates apoptosis of the tumor cells surrounding lumina filled with erythrocytes, as detected by diaminobenzidine colorimetric brown staining of the nuclei. A, PBS-treated control animal tumors; B, angiostatin-treated animal tumors. $\times 200$.

of angiostatin-treated mice was not significantly higher than age-matched control beige nude mice. By contrast, the spleen weight of control, PBS-treated mice was significantly higher than nontumor-bearing control mice ($P < 0.001$; Fig. 2B). The platelet count in mice treated with angiostatin (438 ± 31 K/ μ l) was not significantly lower than nontumor-bearing control mice (606 ± 142 K/ μ l; $P < 0.12$) but was significantly lower in the control, PBS-treated mice (149 ± 21 K/ μ l; $P < 0.0005$; Fig. 2C). Angiostatin also maintained the hematocrit levels ($32 \pm 6\%$) almost to the levels in normal mice without the tumor ($39 \pm 2\%$; $P < 0.15$). In comparison, hematocrit levels in PBS-treated control mice were significantly decreased ($16.7 \pm 0.6\%$; $P < 0.01$ when paired with hematocrits in angiostatin treated mice; Fig. 2D).

Detection of Angiostatin in Plasma 24 h after Injection. Mice were euthanized 24 h after the final injection of angiostatin, and plasma was analyzed by Western blot for the presence of angiostatin. Angiostatin was not detected in the plasma of PBS-treated control mice (Fig. 3), consistent with control studies that indicate that EOMA cells *in vitro* do not produce or secrete angiostatin (data not shown). However, a band the size of the administered human angiostatin (approximately M_r 50,000; Ref. 3) that reacted with antiplasminogen antibody was found in all angiostatin-treated mice (Fig. 3).

Mechanism of Action. There was no significant difference in the proliferative rate between PBS-treated ($19.2 \pm 5.9\%$ of cells) and angiostatin-treated tumors ($22.2 \pm 6.0\%$ of cells) as detected by PCNA staining ($P = 0.08$). By contrast, angiostatin-treated animals demonstrated significantly higher numbers of apoptotic tumor cells ($52.3 \pm 14.6\%$ of cells; Fig. 4A) in comparison with PBS-treated animals ($6.1 \pm 1.9\%$ of cells; Fig. 4B; $P < 0.00001$). *In vitro* studies showed that 2% of EOMA cells in culture were apoptotic, regardless of incubation with angiostatin or the presence or absence of serum. The failure of

angiostatin to induce apoptosis of EOMA cells *in vitro* suggests that the apoptosis observed *in vivo* is the result of angiostatin-induced angiosuppression and supports the model that tumor cell survival is dependent upon factors elaborated by the endothelial cells (1).

Taken together, our data suggest the value of angiostatin administration for treatment of hemangioendothelioma, a primary nonmetastasizing neoplasm, and perhaps of other primary neoplasms. The lack of consistent efficacy of present therapy for KMS, the severe potential side effects, and marked disfigurement in surviving infants after resolution of the massive vascular tumors emphasize the need for more efficacious, less toxic agents to treat KMS. The demonstration that angiostatin not only dramatically inhibits tumor growth but also limits the hematological complications suggests that antiangiogenic agents, in particular angiostatin, may be a primary or adjunctive therapy for KMS.

Acknowledgments

We thank Dr. M. S. Stack for critical review of the manuscript and Deborah Cundiff, Ryan Schultz, and Amy Farmer for expert technical assistance. We appreciate Dr. Susan Crawford's review of the histological sections and Dr. Chong Wang's help with photography.

References

- Folkman, J., and Shing, Y. Angiogenesis. *J. Biol. Chem.*, 267: 10931-10934, 1992.
- O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H., and Folkman, J. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell*, 79: 315-328, 1994.
- Gately, S., Twardowski, P., Stack, M. S., Cundiff, D. L., Grella, D., Castellino, F. J., Enghild, J., Kwaan, H. C., Lee, F., Kramer, R. A., and Soff, G. A. The mechanism of cancer-mediated conversion of plasminogen to the angiogenesis inhibitor, angiostatin. *Proc. Natl. Acad. Sci. USA*, 94: 10868-10872, 1997.
- Dong, Z., Kumar, R., Yang, X., and Fidler, I. J. Macrophage-derived metalloelastase is responsible for the generation of angiostatin in Lewis lung carcinoma. *Cell*, 88: 801-810, 1997.
- O'Reilly, M. S., Holmgren, L., Chen, C., and Folkman, J. Angiostatin induces and sustains dormancy of human primary tumors in mice. *Nat. Med.*, 2: 689-692, 1996.
- Sim, B. K. L., O'Reilly, M. S., Liang, H., Fortier, A. H., He, W., Madsen, J. W., Lpavevich, R., and Nacy, C. A. A recombinant human angiostatin protein inhibits experimental primary and metastatic cancer. *Cancer Res.*, 57: 1329-1334, 1997.
- Wu, Z., O'Reilly, M. S., Folkman, J., and Shing, Y. Suppression of tumor growth with recombinant murine angiostatin. *Biochem. Biophys. Res. Commun.*, 236: 651-654, 1997.
- Holmgren, L., O'Reilly, M. S., and Folkman, J. Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nat. Med.*, 1: 149-153, 1995.
- Esterly, N. B. Kasabach-Merritt syndrome in infants. *J. Am. Acad. Dermatol.*, 8: 504-513, 1983.
- Hoak, J. C., Warner, E. D., Cheng, H. F., Fry, G. L., and Hankenson, R. R. Hemangioma with thrombocytopenia and microangiopathic anemia (Kasabach-Merritt syndrome): an animal model. *J. Lab. Clin. Med.*, 77: 941-950, 1971.
- Folkman, J., Haudenschild, C. C., and Zetter, B. R. Long-term culture of capillary endothelial cells. *Proc. Natl. Acad. Sci. USA*, 76: 5217-5221, 1979.
- Gately, S., Twardowski, P., Stack, M. S., Patrick, M., Boggio, L., Cundiff, D. L., Schnaper, H. W., Madison, L., Volper, O., Bouck, N., Enghild, J., Kwaan, H. C., and Soff, G. A. Human prostate carcinoma cells express enzymatic activity that converts human plasminogen to the angiogenesis inhibitor, angiostatin. *Cancer Res.*, 56: 4887-4890, 1996.
- Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.*, 72: 248-254, 1976.
- Folkman, J. Clinical applications of research on angiogenesis. *N. Engl. J. Med.*, 333: 1757-1763, 1995.
- Hanahan, D., and Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*, 86: 353-364, 1996.
- Enjolras, O., Wassef, M., Mazoyer, E., Frieden, I. J., Rieu, P. N., Drouet, L., Taieb, A., Stalder, J.-F., and Escande, J.-P. Infants with Kasabach-Merritt syndrome do not have "true" hemangiomas. *J. Pediatr.*, 130: 631-640, 1997.
- Ezekowitz, R. A. B., Mulliken, J. B., and Folkman, J. Interferon α -2a therapy for life-threatening hemangiomas of infancy. *N. Engl. J. Med.*, 326: 1456-1463, 1992.
- Chang, E., Boyd, A., Nelson, C. C., Crowley, D., Law, T., Keough, K. M., Folkman, J., Ezekowitz, R. A., and Castle, V. P. Successful treatment of infantile hemangiomas with interferon- α -2b. *J. Pediatr. Hematol. Oncol.*, 19: 237-244, 1997.
- O'Reilly, M. S., Brem, H., and Folkman, J. Treatment of murine hemangioendotheliomas with the angiogenesis inhibitor AGM-1470. *J. Pediatr. Surg.*, 30: 325-330, 1995.

Figure 1. Ex vivo plasma treated with captopril (free sulfhydryl donor) or uPA (urokinase plasminogen activator) and captopril. No plasmin nor plasmin nor AS4.5 was generated by captopril alone. With combined uPA and captopril treatment, plasminogen was activated to plasmin as judged by the formation of the plasmin (PLSMN) complex with alpha-2-antiplasmin (α 2AP) and alpha-2-macroglobulin (α 2MG). Further, the combined uPA/captopril treatment resulted in the generation of significant levels of Lys-AS4.5 (~200 nM) and Glu-AS4.5 (~100 nM).

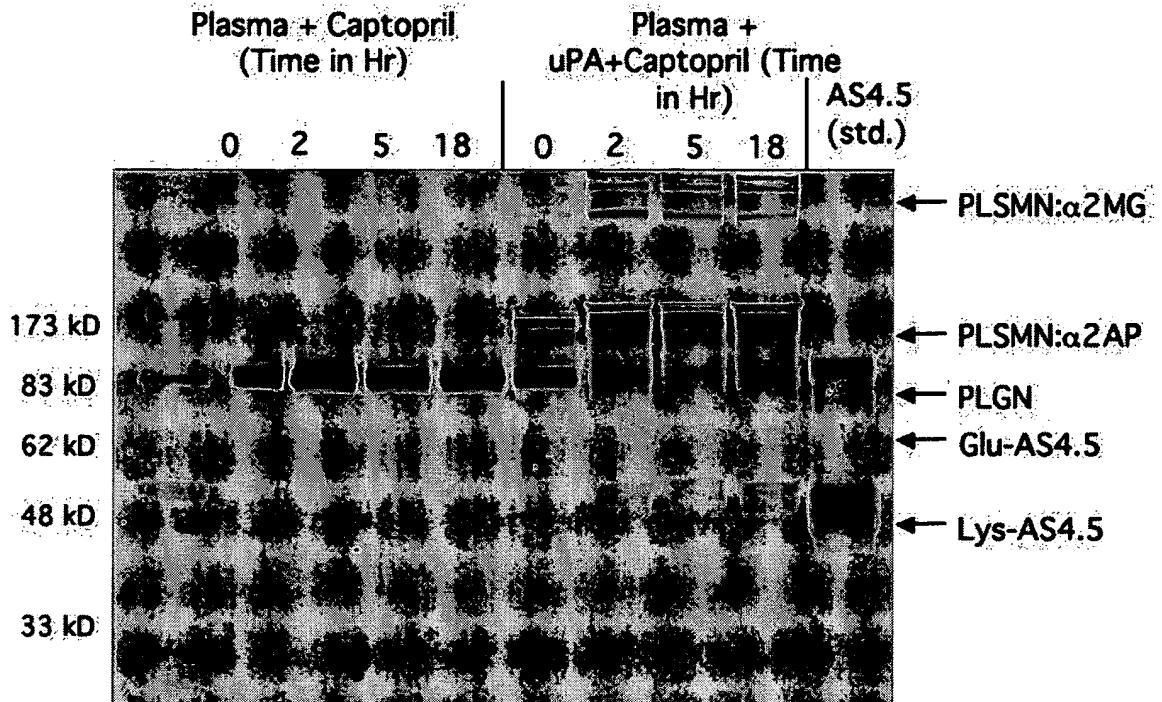
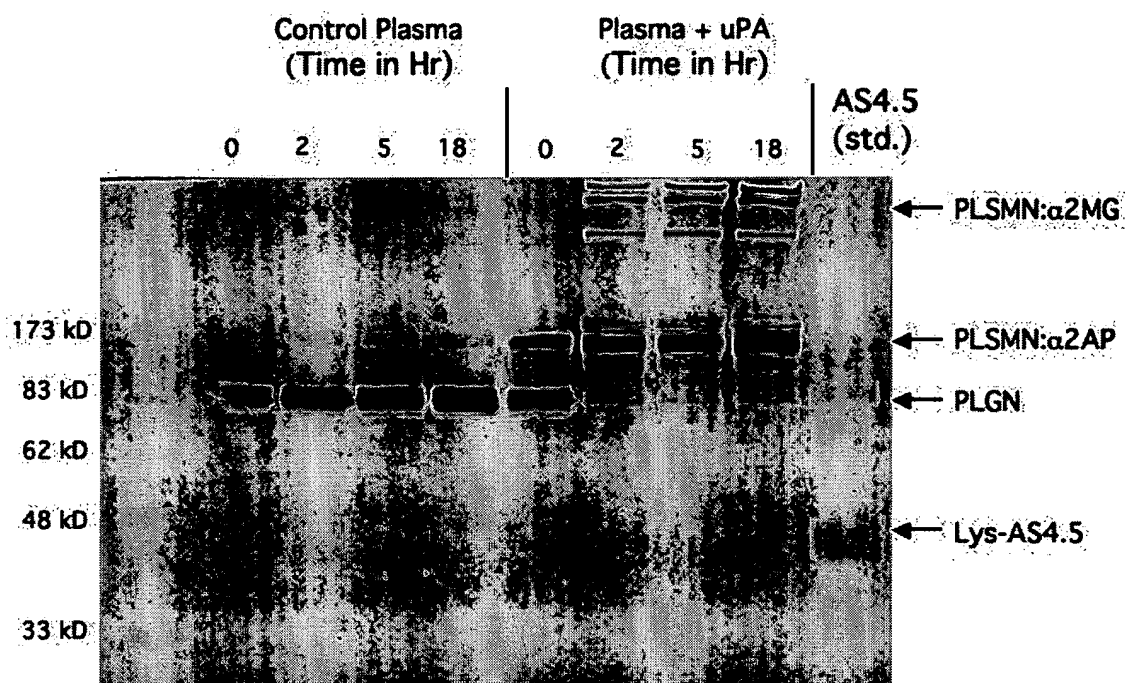


Figure 2 Untreated *ex vivo* plasma (Control) and plasma treated with uPA alone. Neither plasmin nor AS4.5 was generated in untreated control conditions. With uPA treatment alone, however, plasminogen was activated to plasmin as judged by the formation of the plasmin (PLSMN) complex with alpha-2-antiplasmin (α 2AP) and alpha-2-macroglobulin (α 2MG). Further, the uPA treatment resulted in the generation of detectable levels of Lys-AS4.5 (~20-40 nM).



**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.